DESARROLLO DE PRETRATAMIENTOS ENZIMÁTICOS DE MATERIALES LIGNOCELULÓSICOS PARA LA OBTENCIÓN DE BIOETANOL DE SEGUNDA GENERACIÓN

Antonio Pereira González

Sevilla, 2018

Instituto de Recursos Naturales y Agrobiología de Sevilla

Desarrollo de pretratamientos enzimáticos de materiales lignocelulósicos para la obtención de bioetanol de segunda generación

Memoria que presenta:

Antonio Pereira González
para optar al título de Doctor en Ciencias Químicas por la Universidad de Sevilla

Desarrollo de pretratamientos enzimáticos de materiales lignocelulósicos para la obtención de bioetanol de segunda generación

Visado en Sevilla, a 21 de Septiembre de 2018

DIRECTORES

Dra. Dña. Ana Gutiérrez Suárez
Profesora de Investigación del CSIC
IRNAS-CSIC

Dr. D. Jorge Rencoret Pazo
Investigador Contratado
IRNAS-CSIC

Dr. D. José C. del Río Andrade
Profesor de Investigación del CSIC
IRNAS-CSIC

TUTOR

Dr. D. Fernando de Pablos Pons
Catedrático de la Universidad de Sevilla

Memoria que presenta:

Antonio Pereira González
para optar al título de Doctor en Ciencias Químicas por la Universidad de Sevilla

DOCTOR D. JOSÉ ENRIQUE FERNÁNDEZ LUQUE, DIRECTOR DEL INSTITUTO DE RECURSOS NATURALES Y AGROBIOLOGÍA DE SEVILLA DEL CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS.

CERTIFICA: Que la presente Memoria de Investigación titulada "Desarrollo de pretratamientos enzimáticos de materiales lignocelulósicos para la obtención de bioetanol de segunda generación", presentada por Antonio Pereira González para optar al grado de Doctor en Ciencias Químicas, ha sido realizada en el Instituto de Recursos Naturales y Agrobiología del CSIC, bajo la dirección de los Drs. Dña. Ana Gutiérrez Suárez, D. Jorge Rencoret Pazo y D. José C. del Río Andrade, reuniendo todas las condiciones exigidas a los trabajos de Tesis Doctorales.

CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS
INST. RECURSOS NATURALES Y AGROBIOLOGÍA
CSIC
SEVILLA

A mis padres,

a mi hermano y a ti, Api

AGRADECIMIENTOS

Esta Tesis se ha llevado a cabo en el Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS) del Consejo Superior de Investigaciones Científicas (CSIC). Ha sido financiada por los proyectos del Plan Nacional "*Desarrollo de pretratamientos enzimáticos optimizados para la deconstrucción de materiales lignocelulósicos madereros y no madereros*" (LIGNOCELL, AGL2011-25379), y "*Modificación enzimática de lignina y lípidos en las biorrefinerías de la lignocelulosa*" (BIORENZYMERY, AGL2014-53730-R), así como por el proyecto europeo "*Optimized oxidoreductases for medium and large scale industrial biotransformations*" (INDOX, GA-KBBE-2013-7-613549).

Mi más sincero agradecimiento a las personas que tanto directamente como indirectamente han formado parte de este duro trabajo:

A los Dres. Ana Gutiérrez, Jorge Rencoret y José Carlos de Río, directores de esta Tesis, por confiar en mí para trabajar en este grupo y por todos los conocimientos y tiempo que he recibido de ellos. No fue fácil la decisión que tomé, gracias por el apoyo que me disteis. A Ana Gutiérrez por su dedicación y profesionalidad en todo lo que hace, además de su trato personal, amabilidad y simpatía. A José Carlos del Río por su trabajo y por transmitirme sus grandes conocimientos y enseñanzas en el campo de la lignina. Al Dr. Jorge Rencoret, ya que esto es posible gracias a todo el tiempo que me has regalado. Quiero pedirte que continúes luchando por lo que buscas, no hay nadie más capacitado que tú para lo que haces.

A mi hermano científico en la lignina y amigo, el Dr. Alejandro Rico, por tu paciencia para enseñarme todo lo que sabes y por tu alegría todos los días, quiero que sepas que te eché mucho en falta cuando te fuiste.

Al Prof. Ángel T. Martínez, del Centro de Investigaciones Biológicas (CIB-CSIC, Madrid), por su dedicación a los proyectos europeos y por aportar valiosos conocimientos a los artículos en los que he estado implicado.

Al Prof. Fernando de Pablos Pons, tutor de esta Tesis, por haber aceptado la tutoría del mismo, por su amabilidad y ayuda en la parte burocrática.

Al Dr. Orlando Rojas, por la oportunidad de realizar una estancia en su grupo de Carolina del Norte (NCSU), ha sido un gran honor para mí trabajar contigo. A los Drs. Ana Ferrer, Ingrid Hoeguer, Carlos E. Aizpurua y Carlos A. Carrillo por acogerme como uno más, por estar siempre disponibles para mí, por los viajes, por las fiestas…, me hicisteis pasar cuatro meses inolvidables.

A los que han estado conmigo en el día a día, mi grupo de trabajo, Pepijn, Gisela, Carmen y mis compañeros del IRNAS Álvaro, Marco y Antonio, todos habéis aportado un granito de arena a esta Tesis. Quiero agradecer a mis compañeros y amigos, Andrés Olmedo que me ha acompañado desde el inicio de esta Tesis y especialmente al Dr. Esteban Babot, por todas nuestras horas de confidencia y todos los favores sin esperar nada a cambio. Sé que eres una gran persona en todos los sentidos, gracias.

A mis amigos de Huelva, del Colegio Mayor y de Farmacia, he recibido un gran apoyo de vuestra parte, especialmente a Mario Díaz, Alejandro López, Pablo Cabanillas, Diego Marín, Jorge Rueda, Jesús Carbajosa, Ramón Dueñas, Javier Hierro, Miguel Ángel Caracuel, Jaime Saucedo y mi amigo en fatigas Antonio Zurita, he vivido los mejores años de mi vida junto a ustedes. A Sara Font, por apoyarme en el final de esta etapa y acompañarme en el inicio de la siguiente.

A mi familia, por el apoyo que me han dado en todo momento y a aquéllos que he perdido durante estos cinco años, mi abuela Meme y muy especialmente a mi abuelo Api, allá donde estés siempre estarás conmigo.

Especialmente me gustaría dedicar esta Tesis a mis padres, por ellos estoy aquí, por ellos soy lo que soy. Gracias por animarme a no rendirme nunca, por apoyarme en todas las decisiones que tomé, por enseñarme que con trabajo y esfuerzo se pueden conseguir grandes metas, por transmitirme todo en esta vida, en especial el camino a la Virgen del Rocío. Y por último al oftalmólogo Ignacio, mi hermano, un referente para mí por su lucha, esfuerzo y constancia en todo lo que se propone, siento un orgullo grandísimo hacia ti, eres el ejemplo que intento seguir. Los tres sois imprescindibles para mí, os necesito a mi lado.

ABREVIATURAS

ABTS	2,2´-azino-bis(3-etilbenzotializon-6-sulfónico)
AFEX	Expansión de fibra con amoniaco (“Ammonia Fibre Expansion”)
DMSO-d_6	Dimetilsulfóxido deuterado
2D-RMN	Resonancia Magnética Nuclear bidimensional
Ep	Extracción alcalina con peróxido
FA	Ácido ferúlico
FPU	Unidades de papel de filtro (“Filter Paper Units”)
G	Unidad guayacilpropano (o guayacilo)
G´	Unidad guayacilpropano oxidada
H	Unidad 4-hidroxifenilpropano (o 4-hidroxifenilo)
HBT	1- Hidroxibenzotriazol
HSQC	Correlación heteronuclear de cuanto simple (“Heteronuclear Single-Quantum Correlation)
LAS	Lignina ácido soluble
LCC	Complejo lignina-carbohidrato (“Lignin-Carbohydrate Complex”)
LiP	Lignina peroxidasa (“Lignin Peroxidase”)
MeS	Metilsiringato
MnP	Manganeso peroxidasa (“Manganese Peroxidase”)
MtL	Lacasa de *Myceliophthora thermophila*
PCA	Ácido *p*-cumárico
MWL	Lignina aislada de madera molida “Milled Wood Lignin”

PcL	Lacasa de *Pycnoporus cinnabarinus*
QCM	Microbalanza de cristal de cuarzo (“Quarz Crystal Microbalance”)
S	Unidad siringilpropano (o siringilo)
S´	Unidad siringilpropano oxidada
SA	Siringaldehído
SPR	Resonancia de plasmón superficial (“Surface Plasmon Resonance”)
T20	Tween 20
U	Unidad de actividad enzimática

ÍNDICE

RESUMEN

La presente Tesis aborda el estudio de pretratamientos biológicos destinados a romper la barrera de lignina y mejorar la sacarificación de los materiales lignocelulósicos utilizados en la obtención de bioetanol de segunda generación. Los pretratamientos estudiados en esta Tesis están basados en el uso del sistema enzimático lacasa-mediador y han demostrado ser eficaces en degradar/modificar la estructura del polímero de lignina que envuelve y protege a los carbohidratos. Las modificaciones en la estructura de la lignina permiten que los carbohidratos queden más expuestos a las enzimas celulasas, lo que se traduce en una mejora significativa en la hidrólisis de los mismos. Por otro lado, esta Tesis también plantea el estudio de las interacciones que tienen lugar entre la lignina y las celulasas, y que afectan al proceso de hidrólisis de la celulosa. Estos estudios tienen como objetivo un aprovechamiento más racional y eficaz de los materiales lignocelulósicos en la producción de biocombustibles de segunda generación (bioetanol).

En esta Tesis se han evaluado dos sistemas lacasa-mediador. El primero está constituido por una lacasa de alto potencial redox, obtenida del hongo basidiomiceto *Pycnoporus cinnabarinus*, y el mediador redox sintético, 1-hidroxibenzotriazol (HBT). El segundo, incluye una lacasa comercial obtenida del hongo ascomiceto *Myceliophthora thermophila* y el mediador natural siringato de metilo (MeS). Los materiales lignocelulósicos utilizados en los pretratamientos enzimáticos con el sistema lacasa-mediador incluyeron: i) residuos agrícolas de paja de trigo, el bagazo y la paja de la caña de azúcar y ii) madera de paulownia (*Paulownia fortunei*). Para el estudio de las interacciones lignina-celulasa se utilizaron ligninas aisladas de eucalipto (*Eucalyptus globulus*), paja de trigo y pícea (*Picea abies*) .

Los pretratamientos de materiales lignocelulósicos con lacasa de *P. cinnabarinus* en presencia/ausencia de HBT, se realizaron sobre dos materias primas, los residuos agrícolas de paja de trigo y de caña de azúcar, con el objeto de mejorar el rendimiento de sacarificación

enzimática y obtención de bioetanol de ambas materias. Primeramente se realizaron experimentos del tratamiento sobre la paja de trigo en una secuencia que incluía un solo ciclo enzimático seguido de una extracción alcalina con peróxido de hidrógeno. Tras la secuencia se determinó el contenido en lignina (lignina Klason) y se evaluó el efecto que esta eliminación/modificación de lignina producía sobre la sacarificación enzimática (con celulasas). Este pretratamiento produjo un descenso en el contenido en lignina del 45% respecto a la materia prima y una mejora en la obtención de glucosa del 60% tras 72 h de tratamiento con celulasas. También se analizó la modificación de la estructura de lignina con dichos tratamientos mediante resonancia magnética nuclear bidimensional (2D-RMN). Estos estudios revelaron un descenso en los enlaces β-*O*-4´y β-5´ así como en las unidades de lignina *p*-hidroxifenilo (H), guayacilo (G) y siringilo (S), y una disminución más moderada de los ácidos *p*-cumárico y ferúlico, sin variación sustancial en la unidad de tricina.

Con el objeto de estudiar la eficacia del sistema lacasa-mediador sobre otras materias primas, se estudió la aplicación del mismo sobre los residuos de la industria de la caña de azúcar (bagazo y paja), en este caso utilizando una secuencia de 4 ciclos con dosis menores de enzima y mediador por ciclo. Con esta secuencia se obtuvo un descenso en el contenido en lignina del 27% y 31% para el bagazo y paja de caña de azúcar respectivamente, y una mejora del 39% y 46% en el rendimiento de glucosa, en la materia pretratada de bagazo y paja, en comparación con el control, tras las 72 h de tratamiento con celulasas. El análisis 2D-RMN reveló notables cambios en la estructura de la estructura de la lignina, observándose en ambos materiales un descenso significativo en el número los enlaces y unidades de lignina.

La eficacia del sistema lacasa-mediador volvió a quedar demostrada con el pretratamiento realizado sobre madera de paulownia con la lacasa de *M. thermophila* y MeS. Dicho pretratamiento constó de una secuencia de 4 ciclos (cada uno de ellos incluía un tratamiento enzimático seguido de una extracción alcalina). Los mejores resultados se obtuvieron con 50 $U \cdot g^{-1}$ de enzima y 3% de MeS, consiguiendo un descenso en lignina del 24% respecto al control y un aumento en la liberación de glucosa del 40%. Los

análisis de 2D-NMR de estos materiales revelaron un descenso en el número de enlaces y un aumento de las unidades oxidadas de siringilo y guayacilo, tras el pretratamiento. Por último, se analizaron los filtrados obtenidos durante los diferentes ciclos, encontrándose compuestos oxidados de lignina como vainillina, ácido vanílico, siringaldehído y ácido siríngico, que corroboran la naturaleza oxidativa del pretratamiento y la consecuente deslignificación de los materiales lignocelulósicos.

Con el fin de demostrar el efecto negativo que tiene la presencia de lignina sobre las celulasas en la hidrólisis del material lignocelulósico, se aislaron las ligninas (milled wood lignin, MWL) de diferentes materias primas (eucalipto, paja de trigo y pícea), y se estudió su efecto en la adsorción y consecuente inhibición de las celulasas (tanto sobre el cóctel enzimático, como sobre la enzima purificada) mediante microbalanza de cristal de cuarzo y resonancia de plasmón superficial. Los resultados al aplicar las celulasas sobre films recubiertos de dichas ligninas demostraron que la enzima purificada muestra menor afinidad a la adsorción sobre el film con lignina y mayor reversibilidad de unión. También se observó que la afinidad más alta en la adsorción lignina-enzima se produce en la lignina de pícea (99% unidades G) mientras que la adsorción más baja se produjo en la de eucalipto (70% unidades S).

I. INTRODUCCIÓN

Pycnoporus cinnabarinus

I.1. CULTIVOS LIGNOCELULÓSICOS

La biomasa vegetal representa la principal fuente de materiales renovables en la Tierra. Dicha biomasa se encuentra principalmente en las paredes celulares de las plantas y engloba especies tanto agrícolas como forestales de gran interés industrial tanto para la producción de pasta de celulosa, cuya principal fuente son las fibras madereras, como de biocombustibles que se obtienen principalmente a partir de cultivos de origen no maderero debido a su gran disponibilidad y bajo coste. Además, los residuos generados por estos cultivos presentan también gran interés como materia prima para la obtención de celulosa y otros productos.

I.1.1. Cultivos forestales

Están constituidos por diferentes especies de coníferas y frondosas. Las coníferas se utilizan principalmente para la producción de pasta de papel debido a la uniformidad de la madera, la alta resistencia mecánica y la presencia de fibras largas (entre 3 y 5 mm) (García Hortal, 2007). La pícea (**Figura 1**) y el pino son las especies de coníferas más utilizadas en la industria papelera.

Figura 1. Cultivos forestales representativos de coníferas como la pícea (A) y frondosas como el eucalipto (B).

Las frondosas tienen como ventajas el rápido crecimiento y que a partir de ellas se genera un papel con superficie más lisa, bueno para la escritura. Sin embargo, dan lugar a pastas menos uniformes y a un papel más débil. Las especies de frondosas más utilizadas son el eucalipto (**Figura 1**), chopo y abedul. Para la obtención de productos químicos a partir de celulosa como es el caso del bioetanol, las características físicas de la madera no influyen en el producto final pero sí pueden influir en la resistencia a la deconstrucción química de la matriz lignocelulósica.

I.1.2. Cultivos agrícolas

Estructuralmente, las fibras de los cultivos agrícolas son menos densas y más porosas y por tanto, requieren menor energía para su separación. La abundancia de estos cultivos, sumado a su coste relativamente bajo y los ciclos de crecimiento cortos, hacen de ellos una excelente materia prima para la obtención de biocombustibles y celulosa, especialmente en países con escasa disponibilidad de madera.

Las fibras no madereras se pueden clasificar en diferentes categorías. En la primera estarían las fibras procedentes del tallo de plantas como cáñamo, kenaf y lino, o de las hojas, como en el caso del abacá o sisal. En la segunda categoría encontramos los residuos que provienen de los cultivos agrícolas destinados a la alimentación, como la paja de cereales (maíz, trigo, arroz, etc.) o el bagazo de la caña de azúcar (**Figura 2**), que también se utilizan como una fuente importante de material lignocelulósico para obtener biocombustibles de segunda generación (Saini et al., 2015). En la tercera categoría están las hierbas silvestres como el bambú o la hierba elefante, y que se cultivan principalmente para la obtención de biomasa.

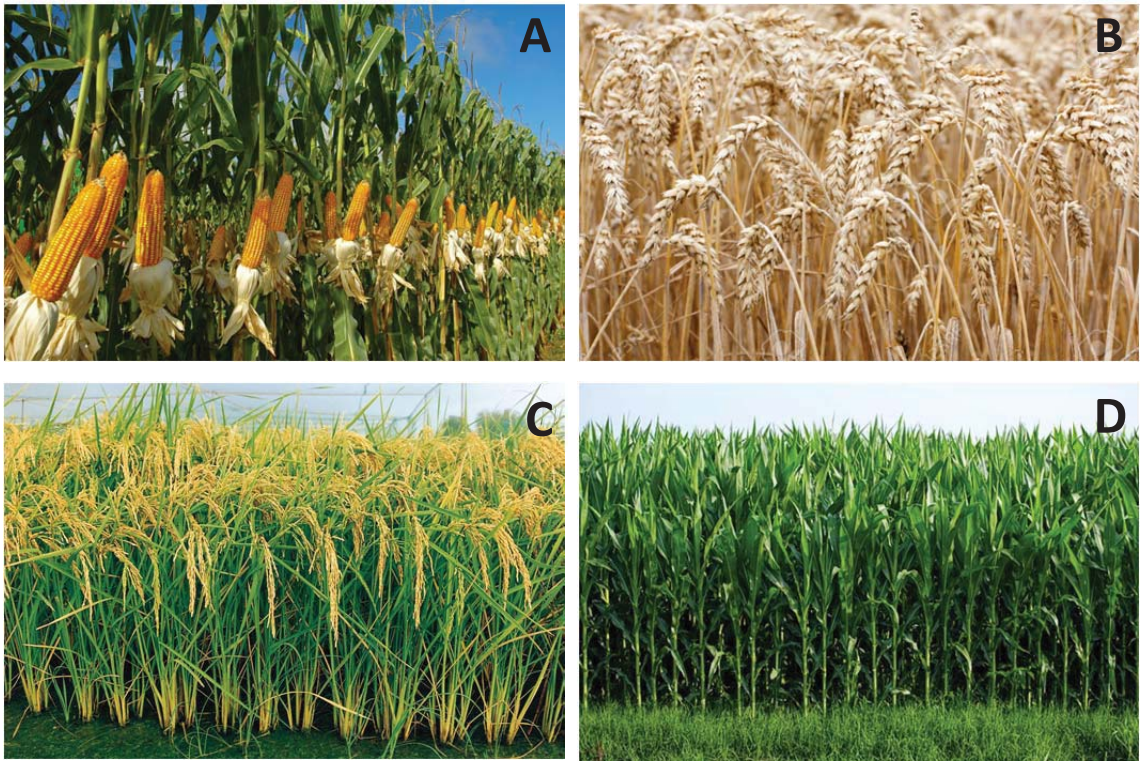


Figura 2. Ejemplos de cultivos agrícolas destinados a alimentación: A) maíz, B) trigo, C) arroz y D) caña de azúcar.

I.2. ESTRUCTURA Y COMPOSICIÓN DE LA LIGNOCELULOSA

Los materiales lignocelulósicos tienen una estructura y composición química muy heterogénea, que condicionan su uso a nivel industrial. Están constituidos mayoritariamente por tres polímeros estructurales presentes en la pared celular vegetal, la celulosa (40-50%), las hemicelulosas (20-30%) y el polímero aromático lignina (10-30%). Además, existen otros compuestos minoritarios de bajo peso molecular, solubles en agua o disolventes orgánicos, y pequeñas cantidades de proteínas y sales minerales (Fengel y Wegener, 1983; Sjöström, 2013).

Las paredes celulares vegetales presentan tres regiones fundamentales, la lámina media (LM), que la conecta con otras células alrededor, una pared primaria (P) y una pared secundaria, la cual puede estar dividida en 2-3 capas, conocidas como S1, S2 y S3 (**Figura 3**). Las capas S1 y S3 son finas, mientras que la capa S2 es de mayor espesor, conformando la mayor

parte de la pared celular tanto en frondosas como en coníferas. Estas capas se diferencian por la orientación de las microfibras de celulosa, en la capa S1 están orientadas horizontalmente y en la S2, las microfibras se orientan casi verticalmente. Dicha orientación, sumado al grosor de la capa S2, hacen que esta capa confiera tanto la resistencia como las propiedades mecánicas y físicas a la planta (Ek et al., 2009).

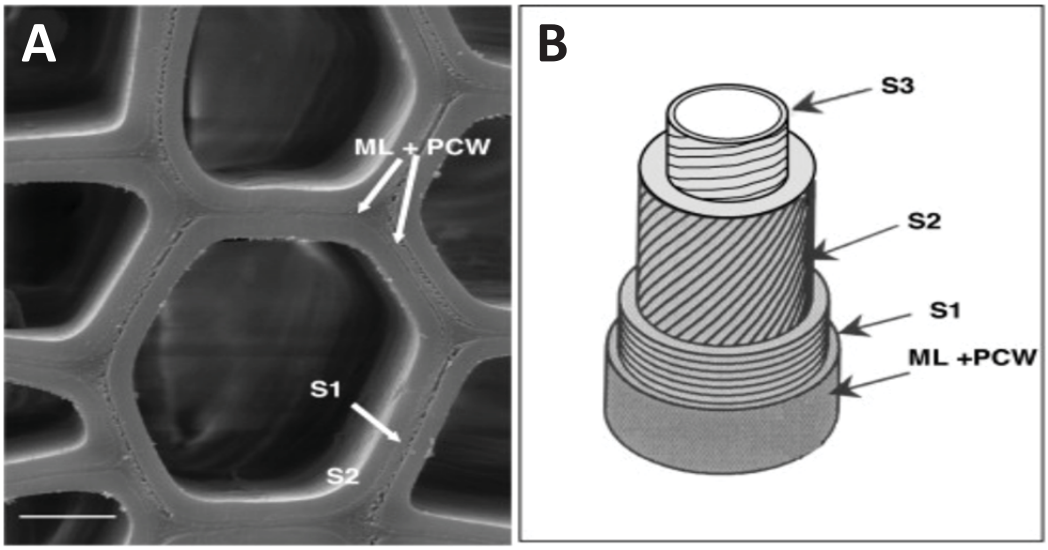


Figura 3. Pared celular de fibras de madera de chopo. A) Microscopía electrónica de barrido de la pared celular vegetal, B) esquema de las diferentes capas de la pared celular vegetal. ML: Lámina media; PCW: Pared celular primaria; S1, S2, S3: Capas de la pared celular secundaria. La escala microscópica corresponde 5 µm. (Adaptado de Déjardin et al., 2010)

La composición química de estas paredes varía ampliamente entre los diferentes tipos de células, entre los diferentes tejidos y entre las diferentes especies de plantas (Zeng et al., 2014).

I.2.1. Celulosa

La celulosa es el componente mayoritario de la pared celular vegetal y tiene como principal función el mantenimiento de la estructura de la planta. El porcentaje de celulosa varía entre los distintos materiales lignocelulósicos, y generalmente representa el 30-50% del peso seco de la planta (Foyle et al., 2007; Harris y DeBolt, 2010).

Estructuralmente, la celulosa es un polímero lineal muy ordenado, constituido por unidades de D-glucosa unidas por enlaces glicosídicos β (1→4). La unidad que se repite es la celobiosa, un disacárido formado por dos unidades de glucosa unidas por el grupo hidroxilo del carbono 1 en posición β de una glucosa y el grupo hidroxilo del carbono 4 de la otra (**Figura 4**), con la eliminación de una molécula de agua. La formación de este tipo de enlace β implica que cada unidad de glucosa este girada 180° con respecto a la anterior, permitiendo la formación de puentes de hidrógeno intramoleculares y dando como resultado largas cadenas de rectilíneas estabilizadas (Fengel y Wegener, 1983; Sjöström, 2013).

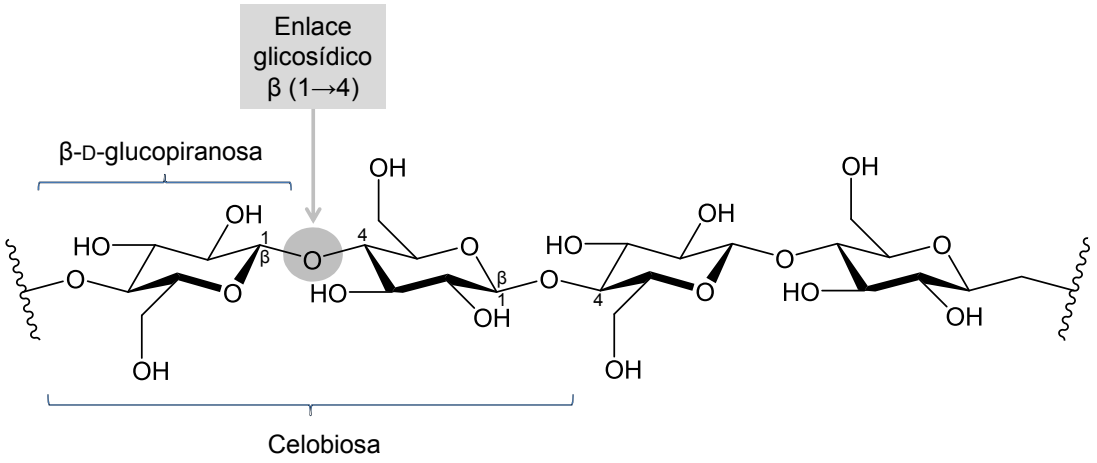


Figura 4. Estructura de la cadena de celulosa.

La molécula de celulosa tiene una fuerte tendencia a formar enlaces por puentes de hidrógeno, tanto entre unidades de glucosa de la misma cadena (intramoleculares), como entre cadenas adyacentes (intermoleculares), siendo estas uniones las que dan lugar a las miofibrillas, cuya unión formará la fibra de celulosa, y cuyos agregados forman la pared celular (**Figura 5**). La existencia de estos enlaces tiene un efecto importante en la reactividad que presentan las cadenas celulósicas. Los enlaces de hidrógeno intermoleculares permiten una estructura fibrilar terciaria de alta cristalinidad. Las zonas que presentan elevada cristalinidad son difíciles de penetrar por disolventes y reactivos. Por el

contrario, las zonas más desordenadas (amorfas), son más accesibles y más susceptibles a toda reacción química.

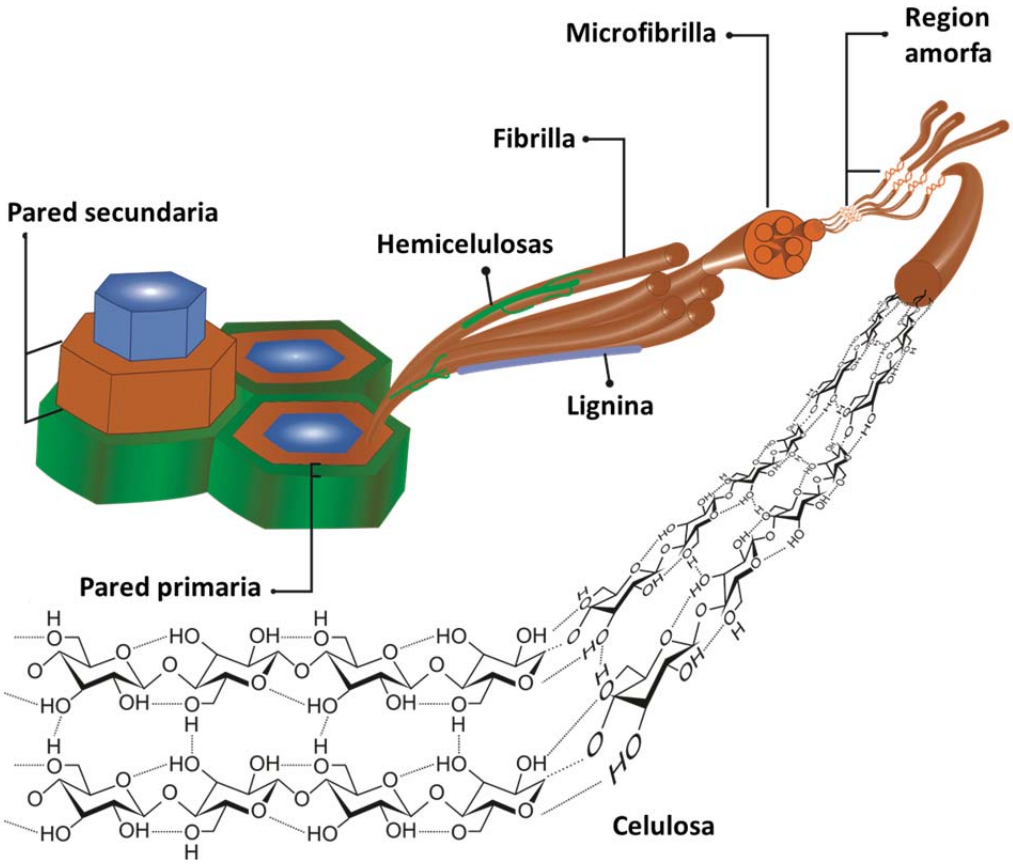


Figura 5. Estructuración de las cadenas de celulosa en micro y macrofibrillas.

I.2.2. Hemicelulosas

Al contrario que la celulosa, las hemicelulosas son un grupo de polisacáridos químicamente heterogéneo, compuesto por diferentes unidades de monosacáridos incluyendo pentosas (D-xilosa y L-arabinosa), hexosas (D-glucosa, D-galactosa, D-manosa, L-ramnosa y L-fucosa) y ácidos urónicos (ácido D-glucurónico y ácido D-galacturónico) (**Figura 6**). Estos monosacáridos se encuentran unidos principalmente por enlaces β (1→4) y en algunos casos β (1→3) formando estructuras ramificadas y generalmente amorfas (Sjöström y Westermark, 1999; Scheller y Ulvskov 2010).

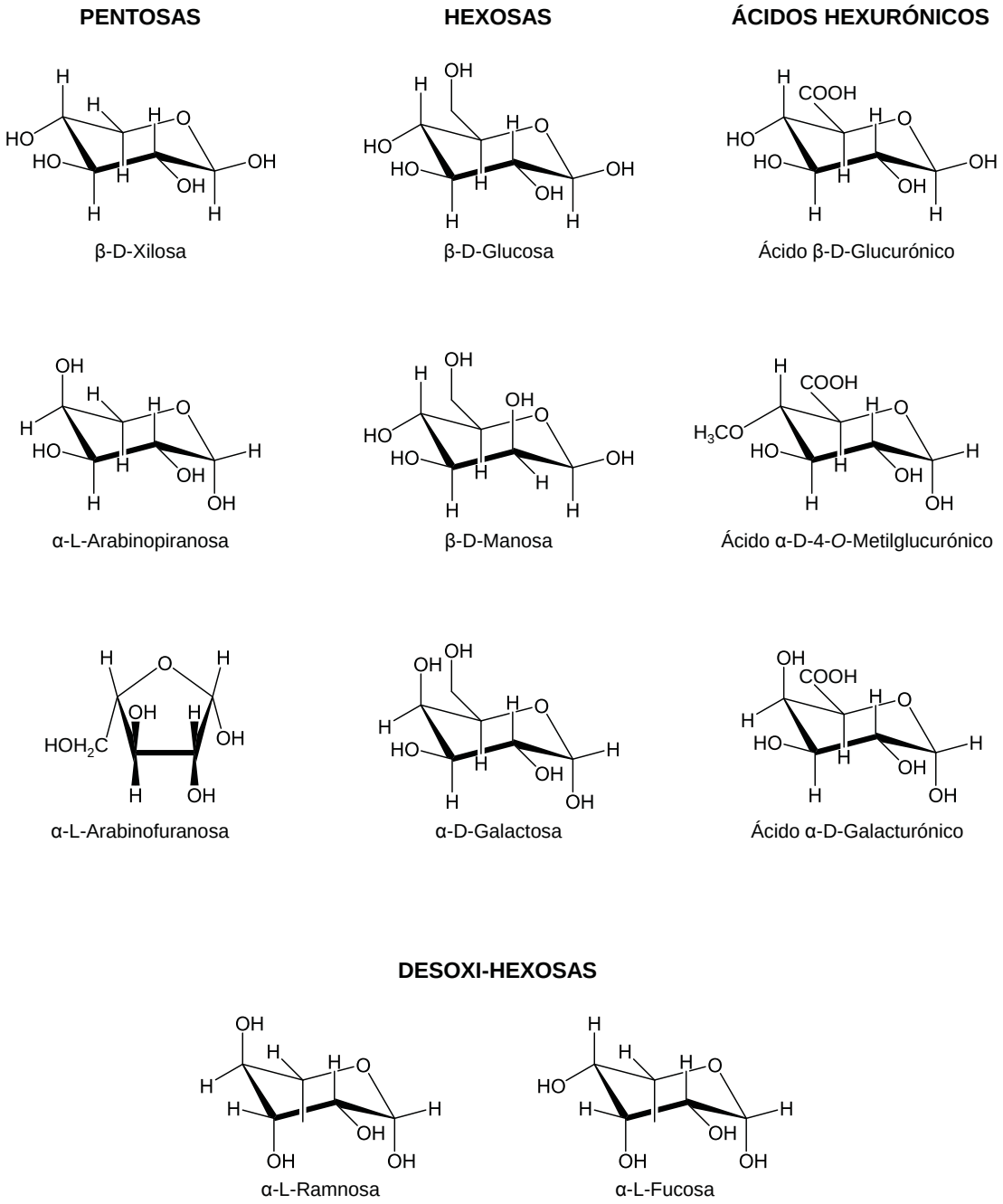


Figura 6. Monosacáridos presentes en las hemicelulosas (adaptado de Fengel y Wegener, 1984)

Las hemicelulosas representan un 25-30% del peso seco de las maderas de coníferas y un 20-43% de la madera de frondosas (Aitken et al., 1988). Pueden estar asociadas tanto a la porción celulósica como a la lignina y su función, al igual que la celulosa, es de soporte aunque son más fácilmente accesibles y degradables por la acción de ácido diluido, base diluida o las

enzimas hemicelulasas, que eliminan tanto las cadenas laterales como la cadena principal aleatoriamente, para liberar oligosacáridos que serán degradados en azúcares simples (Abdel-Hamid et al., 2013).

I.2.3. Lignina

La lignina es, tras la celulosa, el polímero más abundante en la superficie terrestre, se encuentra en la mayoría de plantas terrestres en las que representa entre el 25-33% de la biomasa seca en maderas de coníferas y entre el 18-34% en la madera de frondosas (Boerjan et al., 2003; Ragauskas, 2014). La lignina realiza diversas funciones entre las que destacan su papel en el transporte interno de agua, metabolitos y nutrientes y proporciona una superficie hidrófoba al sistema vascular de la planta. También tiene función como aglomerante de las fibras de celulosa y hemicelulosas debido a su carácter hidrófobo, proporcionando rigidez y resistencia a la pared celular, así como protección ante agentes patógenos (Sarkanen y Ludwig, 1971; Chandra et al., 2007).

Estructuralmente, la lignina es un heteropolímero aromático, muy ramificado y amorfo. Está constituido por unidades de fenilpropano con diferentes patrones de sustitución unidos por diferentes enlaces, que varían considerablemente entre las diferentes especies vegetales (Ralph et al., 2004; Boerjan et al., 2003; Vanholme et al., 2010).

La lignina se sintetiza a partir del aminoácido fenilalanina mediante la ruta del ácido cinámico (**Figura 7**) (Freudenberg y Neish, 1968; Adler, 1977; Boerjan et al., 2003; Higuchi, 2012). Una serie de enzimas son las encargadas de la conversión del L-fenilalanina en los alcoholes *p*-cumarílico, coniferílico y sinapílico que actúan como precursores o monómeros de la lignina. Estos alcoholes *p*-hidroxicinamílicos, también conocidos como monolignoles, solo se diferencian estructuralmente en el número de metoxilos presentes sus anillos aromáticos (**Figura 7**).

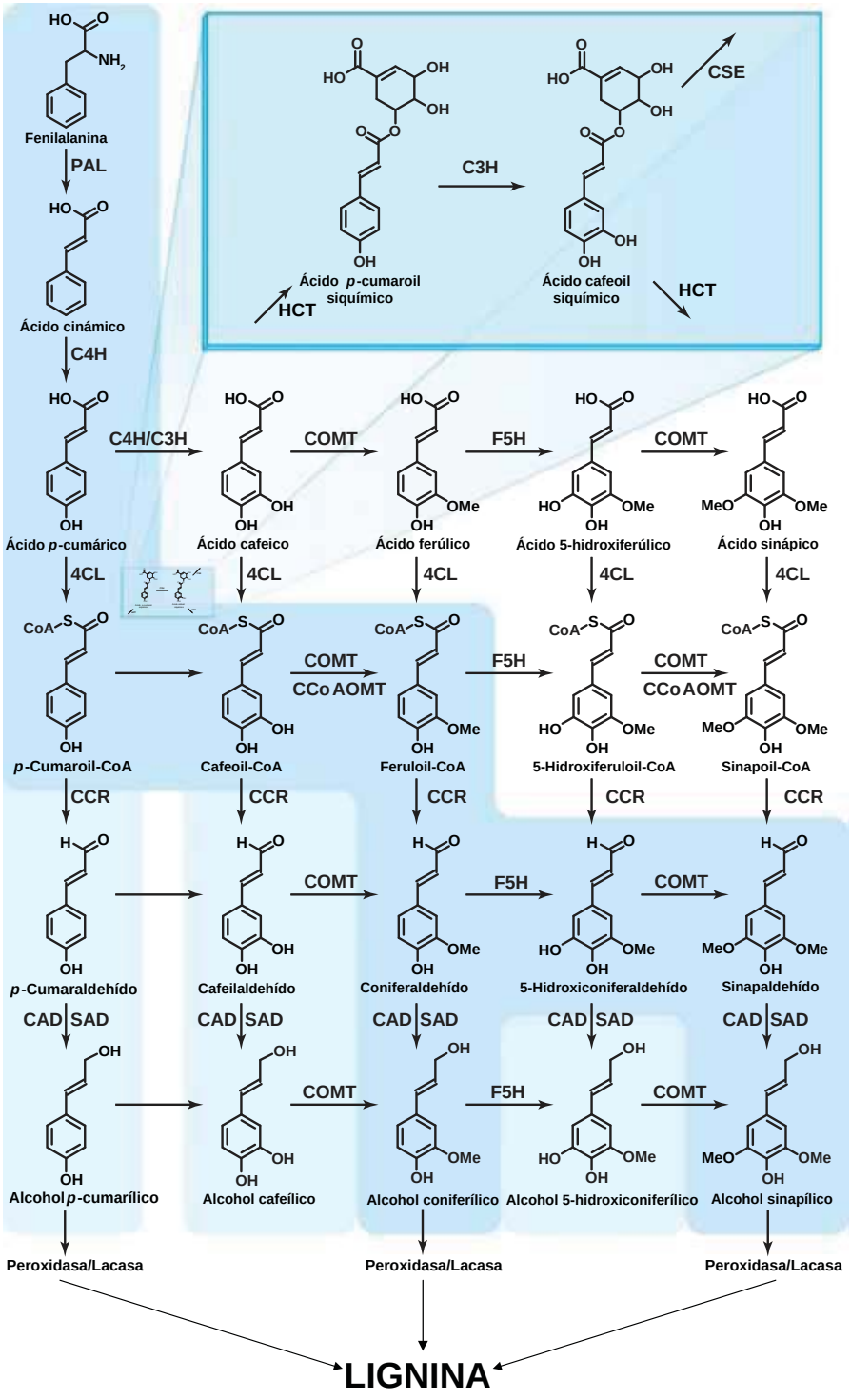


Figura 7. Ruta biosintética de la lignina (adaptada de Boerjan et al., 2003). Existen distintas rutas, pero la ruta de color azul es la más favorecida. En la ruta intervienen once enzimas, que en orden de actuación son las siguientes: amonio liasa (PAL), cinamato-4-hidroxilasa (C4H), 4-cumarato CoA ligasa (4CL), cafeico *O*-metiltransferasa (COMT), cinamoil CoA-reductasa (CCR), cafeoil CoA-3-*O*-metiltranferasa (CCoAOMT), cinamil alcohol deshidrogenasa (CAD), ferulato-5-hidroxilasa (F5H), sinapil alcohol deshidrogenasa (SAD), *p*-cumarato-3-hidroxilasa (C3H) y caffeoyl shikimate esterase (CSE).

Estos monolignoles se incorporan a la lignina y dan lugar a las distintas unidades de *p*-hidroxifenilo (H), guayacilo (G) y siringilo (S) (**Figura 8**). La composición de la lignina en términos H:G:S varía ampliamente en la biomasa vegetal dependiendo de la especies. En términos generales, la lignina de plantas herbáceas está constituida por las tres unidades monoméricas G, S y H, la lignina de frondosas contiene unidades G y S en distinta proporción y la lignina de coníferas está básicamente compuesta por unidades G (Abdel-Hamid et al., 2013). La composición de la lignina también varía con la edad de la planta (Freudenberg y Neish, 1968; Rencoret et al., 2010), el lugar de la pared celular o lámina media donde se sintetice (Fukushima y Terashima, 1991; Christiernin et al., 2005) y el tejido de la misma (Bland 1966; Hardell et al., 1980a,b; Lourenço et al., 2016).

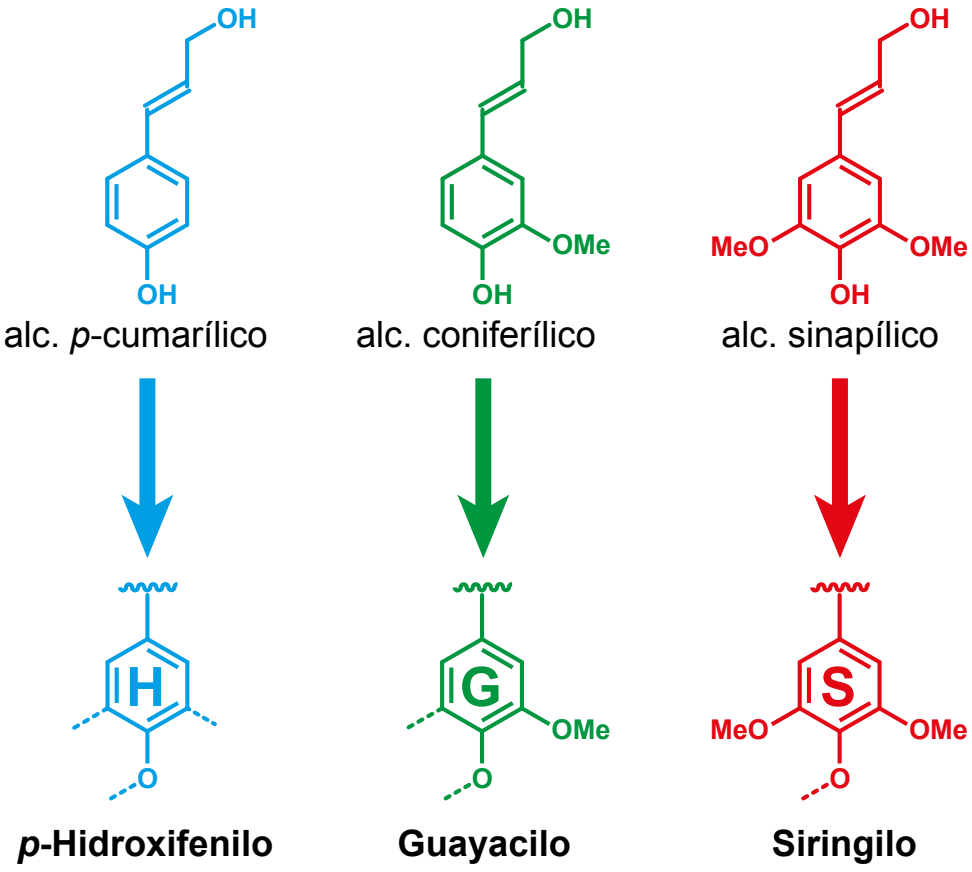


Figura 8. Unidades *p*-hidroxifenilo (H), guayacilo (G) y siringilo (S) de la lignina, que se forman tras la polimerización de los respectivos monolignoles, alcohol *p*-cumarílico, coniferílico y sinapílico (adaptado de Ralph et al., 2004).

Las unidades S de lignina tienen dos grupos metoxilos en las posiciones C-3 y C-5 por lo que no pueden establecer enlaces condensados carbono-carbono en el anillo. Principalmente se unen a otras unidades mediante enlaces de tipo éter a través del grupo hidroxilo de su C-4 y, por lo tanto, forman ligninas con una estructura lineal y más fácil de degradar. Por el contrario, las unidades H tienen libres los C-3 y C-5, ya que no poseen grupos metoxilos, por lo que consecuentemente estas unidades pueden formar enlaces C-C en estas posiciones y formarán ligninas con mayor grado de condensación. Las unidades G se encuentran en un término intermedio ya que tienen ocupada la posición C-3 por un grupo metoxilo y la C-5 libre y disponible para la formación de enlaces carbono-carbono, por lo que las ligninas ricas en unidades G serán más difíciles de degradar que aquellas enriquecidas en unidades S (del Río et al., 2005).

Además de los tres monolignoles tradicionales, se han descrito otros compuestos fenólicos que también actúan como monómeros de la lignina participando en reacciones de acoplamiento durante la lignificación. Entre ellos se encuentran los derivados acilados de los correspondientes alcoholes *p*-hidroxicinamílicos con acetatos, *p*-cumaratos, *p*-hidroxibenzoatos, ferulatos, benzoatos y vainillatos (**Figura 9A**) (del Río et al., 2007, 2008; Martínez et al., 2008; Rencoret et al., 2013, Karlen et al. 2017), compuestos intermediarios de la ruta biosintética de lignina como el aldehído cinamílico, ácido ferúlico, alcohol cafeílico y alcohol 5-hidroxiconiferílico (**Figura 9B**) (Kim et al., 2003; Leplé et al., 2007; Chen et al., 2013) e incluso compuestos fenólicos procedentes de otras rutas biosintéticas (flavonoides y estilbenoides). Estos últimos han sido descubiertos recientemente, el primero de ellos fue la flavona tricina, presente en las ligninas de gramíneas y otras monocotiledóneas (del Río et al., 2012), y recientemente se han descrito los hidroxiestilbenos piceatanol, isorhapontigenina y resveratrol (**Figura 9C**), presentes en las ligninas de los endocarpos de frutos de palmas (del Río et al., 2017; Rencoret et al., 2018).

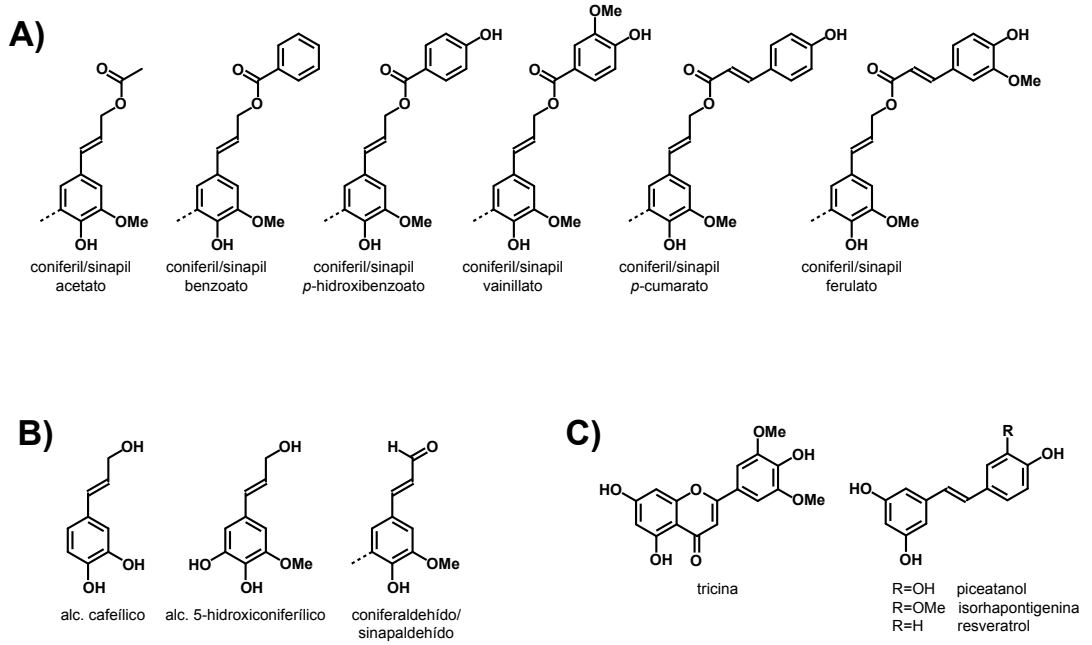


Figura 9. Otros monómeros encontrados en el polímero de lignina. A) alcoholes *p*-hidroxicinamílicos γ-acilados, B) compuestos intermediarios de la ruta biosintética de la lignina, C) compuestos que proceden de otras rutas biosintéticas (flavonoides y estilbenoides).

La lignificación comienza con la deshidrogenación enzimática (mediante peroxidasas y/o lacasas) de los monolignoles, que da lugar a la formación de radicales libres tipo fenoxilo, estabilizados por resonancia (**Figura 10**).

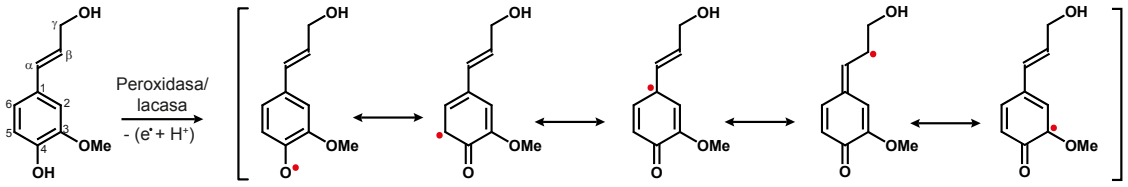


Figura 10. Deshidrogenación enzimática del alcohol coniferílico y formas resonantes de radical fenoxilo (adaptado de Adler, 1977).

A continuación, tiene lugar el acoplamiento de los radicales entre sí y con el polímero creciente de lignina mediante diversos tipos de enlaces. Las uniones que se producen se pueden clasificar en dos grandes grupos, enlaces de tipo éter y uniones de tipo carbono-carbono (**Figura 11**). Las

uniones tipo éter incluyen los enlaces β-*O*-4 y 4-*O*-5, siendo el enlace β-*O*-4 el más común en la lignina. Las uniones carbono-carbono, también conocidas como enlaces condensados, incluyen los enlaces β-β resinol, formados por la unión de dos cadenas alifáticas, los enlaces β-1, al unirse un carbono de una cadena alifática con un carbono de un anillo bencénico de otra unidad, formando una estructura de tipo espirodienona (Zhang y Gellerstedt, 2001), enlaces β-5 fenilcumaranos, y enlaces 5-5, formados por la unión de carbonos de anillos bencénicos, que se encuentra en forma de trímero, al incorporarse una tercera unidad mediante enlaces α-*O*-4′/β-*O*-4′′ dando lugar a una estructura de tipo dibenzodioxocina (Karhunen et al. 1995). La proporción de estos enlaces varía en las diferentes especies de plantas, siendo el β-*O*-4 el más abundante.

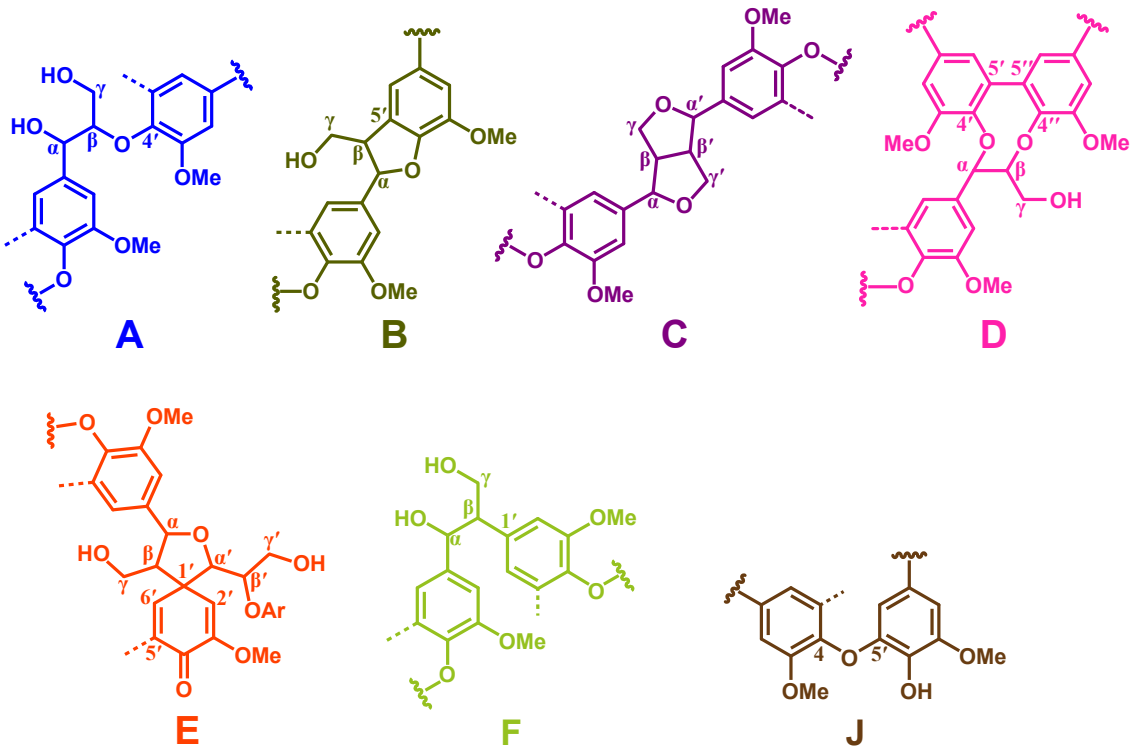


Figura 11. Principales subestructuras presentes en la lignina. A) β-O-4 alquil-aril éter, B) β-5 fenilcumarano, C) β-β resinol, D) 5-5 dibenzodioxacina, E) β-1 espirodienona, F) β-1, J) 4-O-5 aril-aril éter.

El conocimiento en la estructura de la lignina ha avanzado enormemente en los últimos años gracias a los avances en las técnicas analíticas,

especialmente en la 2D-NMR. En la **Figura 12** se muestra un modelo estructural de las ligninas de coníferas y de frondosas.

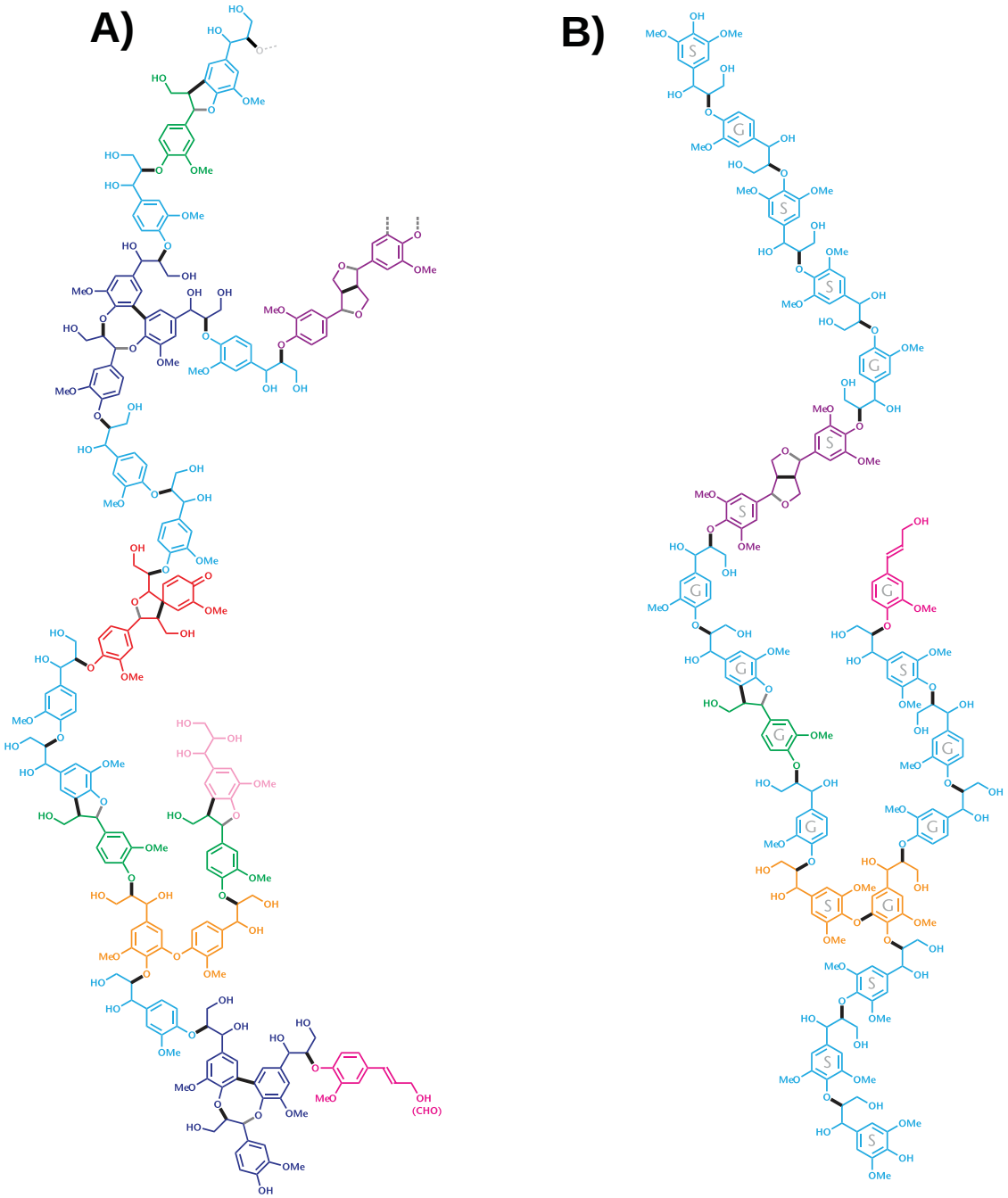


Figura 12. Modelo del polímero de lignina en madera: A) conífera (pícea) (Brunow, 2005) y B) frondosa (álamo) (Boerjan et al., 2003).

La lignina también se asocia a los polisacáridos mediante enlaces covalentes formando los llamados complejos macromoleculares lignina-

carbohidrato (**Figura 13**). Existen tres tipos de enlaces lignina-carbohidrato, glucósidos de fenilo, ésteres del ácido 4-*O*-metilglucurónico con el C_{γ} de la lignina y ésteres de bencilo (Fengel y Wegener, 1983; Balakshin et al., 2011; del Río et al., 2016).

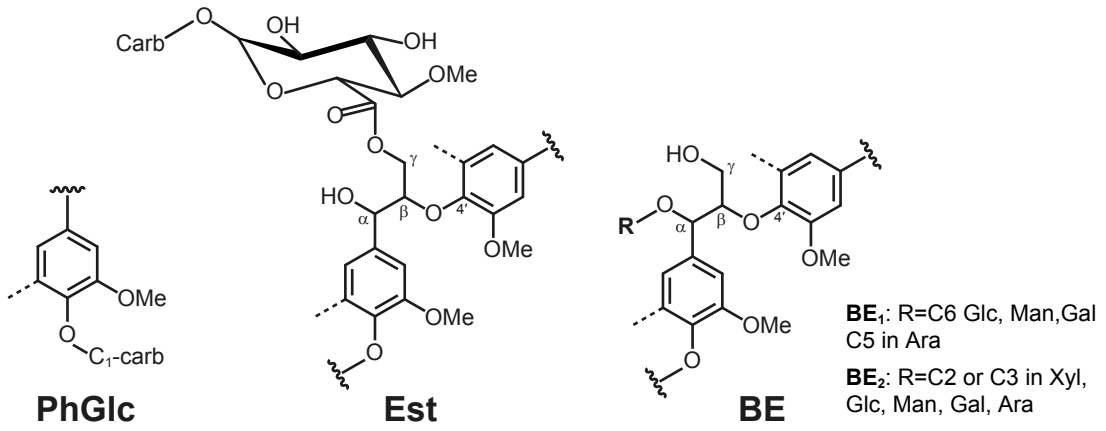


Figura 13. Principales uniones lignina-carbohidrato observadas en plantas: fenil glicósidos (PhGlc), lignina carbohidrato-ester (Est) y bencil éteres (BE).

I.2.4. Compuestos minoritarios

Además de la celulosa, las hemicelulosas y la lignina, existen otros componentes que se presentan en menor proporción y entre los cuales se encuentran los extraíbles hidrofílicos y lipofílicos, proteínas, pectinas y compuestos inorgánicos (Sun, 2010). Estos compuestos minoritarios no influyen en la estructura morfológica de la pared celular, pero desempeñan funciones vitales para la célula vegetal como la protección a insectos. Además son los responsables del color y olor de la madera. Sin embargo, algunos de ellos tienen un efecto negativo en la producción de la pasta de papel, como por ejemplo la formación de depósitos de pitch en la fabricación de la pasta de celulosa producida por algunos extraíbles lipofílicos (Gutiérrez et al., 2001, 2006).

Extraíbles hidrofílicos: engloban compuestos fenólicos libres de bajo peso molecular, que se pueden aislar del material lignocelulósico mediante extracciones con disolventes polares. Se incluyen dentro de este grupo precursores de lignina (ácidos *p*-hidroxicinamílicos y aldehídos *p*-

hidroxicinámicos), ácidos bencenocarboxílicos relacionados (ácido *p*-hidroxibenzoico, vainíllico y siríngico), aldehídos y cetonas aromáticas (*p*-hidroxibenzaldehído, vainillina y siringaldehído y propioguayacona), lignanos (dilignoles y compuestos relacionados), taninos hidrolizables (ésteres de ácido gálico y sus dímeros), taninos no hidrolizables (varias unidades de flavonoides condensadas) y flavonoides (estructura derivadas del anillo de flavona).

Extraíbles lipofílicos: incluyen alcanos, alcoholes grasos, aldehídos, ácidos grasos, esteroles libres y conjugados, ácidos resínicos, ceras (ésteres de ácido graso con alcoholes de cadena larga) y glicéridos (ésteres de ácido graso con glicerol). Se pueden obtener estos extraíbles lipófilos del material lignocelulósico con disolventes apolares.

I.3. BIORREFINERÍAS DE LA LIGNOCELULOSA

El concepto de biorrefinería de la lignocelulosa se basa en aprovechar íntegramente esta biomasa vegetal renovable para producir bioenergía y biomateriales mediante la aplicación de procesos físicos, químicos y/o biológicos. Los principales usos industriales son la obtención de celulosa para la producción de pasta de papel y la obtención de biocombustibles de segunda generación (bioetanol), así como la valorización de la lignina, que suele ser un residuo en los procesos anteriores.

El objetivo actual de la biorrefinería es conseguir el *"zero waste",* este término hace referencia al uso eficiente de todos los azúcares y carbonos unidos en la biomasa lignocelulósica (Young et al., 2010). La **Figura 14** muestra el uso que se le da a los distintos componentes mayoritarios del material lignocelulósico.

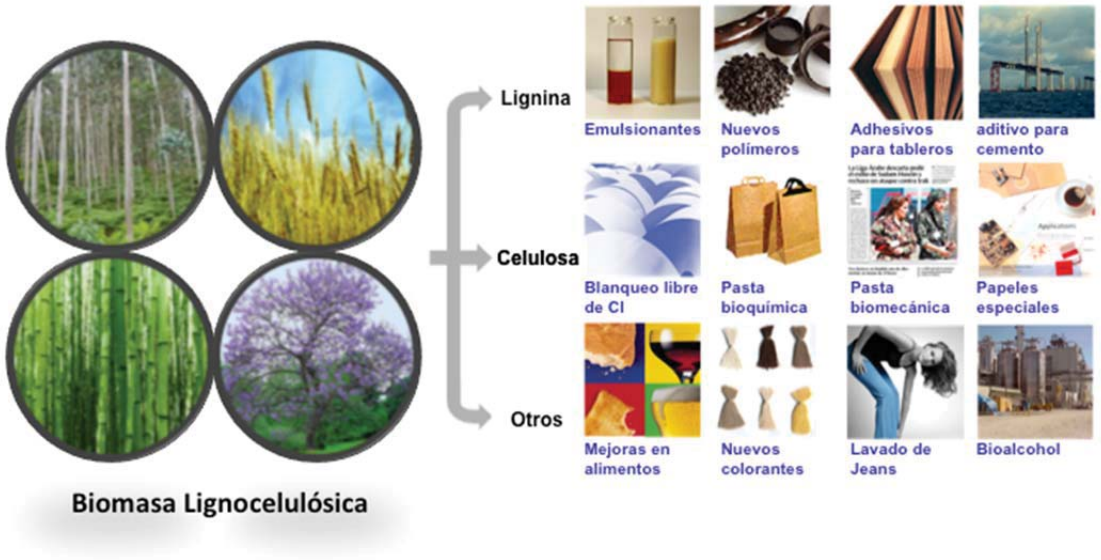


Figura 14. Representación esquemática de usos de la celulosa, hemicelulosa y lignina en la industria.

Los principales usos industriales de la biomasa lignocelulósica se han centrado principalmente en la obtención de la celulosa para la producción de pasta de papel y de biocombustibles. En estos procesos la lignina es un residuo que se quema para generar calor y electricidad. Sin embargo, la lignina también puede ser utilizada para obtener productos de mayor valor añadido (**Figura 15**) como fibra de carbono, plásticos y elastómeros termoplásticos, espumas y membranas poliméricas (Ragauskas, 2014; Ten y Vermerris, 2015).

Algunas fracciones de lignina se usan para obtener combustibles y productos químicos que actualmente provienen del petróleo. Otras fracciones son utilizadas para otras aplicaciones en una gran variedad de industrias como la médica, la agrícola, la industria de los combustibles y la papelera (**Figura 15).**

Figura 15. Aplicaciones o usos que tiene la lignina y sus derivados

I.3.1. Producción de pasta de papel

La producción de pasta de papel consiste básicamente en la separación de las fibras de celulosa de la madera u otros materiales fibrosos, que se encuentran cementadas por la lignina, a través de procesos mecánicos y/o químicos (Fengel y Wegener 1983; Sjöström, 2013).

La obtención de celulosa se basa principalmente en dos procesos, pasteado y blanqueo. El objetivo del pasteado es la separación de las fibras de celulosa del resto de componentes de la madera mediante la destrucción o debilitamiento de los enlaces interfibras a través de procesos mecánicos (utilizando molinos y refinadores de disco) y/o

químicos, en condiciones alcalinas, como el pasteado a la sosa (proceso kraft), o en condiciones ácidas como el pasteado al sulfito. Existen otros tipos de procesos como el proceso organosolv, que utiliza solventes orgánicos.

La mayoría de las pastas de celulosa obtenidas del pasteado son muy oscuras para ser utilizadas. Estas se someten a un proceso de blanqueo en el que se trata químicamente la pasta de celulosa para eliminar las sustancias responsables del color, básicamente la lignina residual que permanecen en la pasta. El blanqueo de las pastas químicas se realiza con secuencias libres de cloro elemental ("*elemental chlorine free*", ECF) utilizando dióxido de cloro o usando secuencias totalmente libres de cloro ("*totally chlorine free*", TCF), utilizando oxígeno, ozono y peróxido de hidrógeno, que son más respetuosos con el medio ambiente. Los métodos de blanqueo de pastas mecánicas, que tienen mayor porcentaje de lignina, se realiza mediante la destrucción de los grupos cromóforos responsables del color, pero sin una eliminación significativa de lignina; los productos químicos utilizados son peróxido de hidrógeno como oxidante e hidrosulfitos como reductores (García Hortal, 2007).

I.3.2. Producción de biocombustibles de segunda generación

Los biocombustibles obtenidos a partir de la biomasa vegetal presentan un gran interés industrial debido a que son una alternativa prometedora a los combustibles fósiles. Se ha predicho que la reserva de combustibles fósiles se agotará en los próximos 40-50 años debido al incremento de consumo de estos combustibles no renovables (Vohra, 2014). Más importante aún son los efectos que produce la combustión de éstos, con la producción de gases de efecto invernadero que contribuyen al calentamiento global (Vanhala et al., 2016). El uso de biocombustibles ayudará a reducir la emisión de gases de efecto invernadero y a mitigar el cambio climático, y por otro lado reducirá la dependencia energética de los países con escasos recursos de petróleo. La producción global de bioetanol está aumentando (**Figura 16**). El material lignocelulósico representa una opción prometedora como materia prima para la producción de etanol considerando su relación de producción / energía de

entrada, disponibilidad, bajo costo y mayores rendimientos de etanol (Saini et al. 2015).

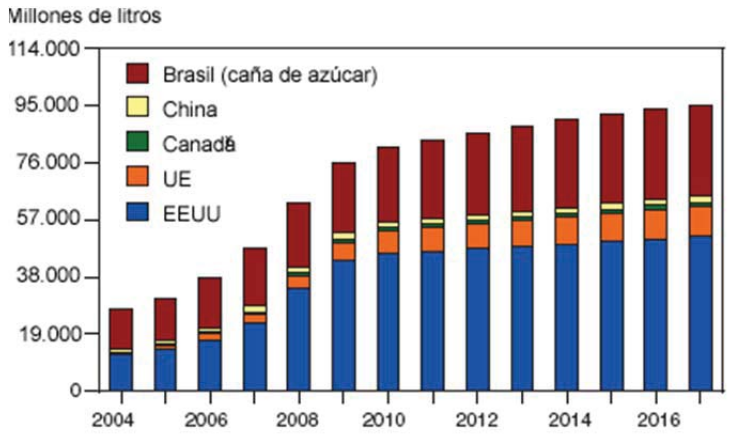


Figura 16. Gráfico que muestra la producción global de bioetanol en los últimos diez años.

Hasta la fecha, numerosos recursos de biomasa han sido investigados para la producción de bioetanol, que pueden clasificarse en los que usan azúcares y almidón de diversos cultivos para uso alimenticio, como son la caña de azúcar o el maíz (denominado bioetanol de primera generación), los que usan materiales lignocelulósicos, como maderas o paja de cereales (bioetanol de segunda generación) y aquéllos que usan algas (bioetanol de tercera generación), cuyo desarrollo está en sus etapas iniciales.

Los biocombustibles de primera generación provienen de cultivos agrícolas destinados a la alimentación humana, entre los que destacan el maíz, la caña de azúcar, el trigo, la remolacha azucarera y la soja. Las tecnologías de producción de biocombustibles de primera generación son más simples y económicas, pero tienen el grave problema de la inseguridad alimentaria que genera debido a los problemas de precios de los alimentos para los sectores más pobres de la población.

Para paliar el problema de la competencia alimentaria, se planteó la producción de biocombustibles a partir de los carbohidratos de la biomasa lignocelulósica. Esta biomasa procede tanto de cultivos energéticos

cultivados para este fin así como de residuos de la actividad agrícola de las industrias alimentaria y forestal. A diferencia de los de primera generación, estos residuos agrícolas y forestales no sólo no tienen valor económico en el contexto en el que se generan, sino que no suelen provocar problemas ambientales durante su eliminación. Los cultivos son abundantes y de rápido crecimiento en ciclos cortos, por lo que las tierras se pueden recuperar fácilmente para el uso que se considere o dedicarlos específicamente a la producción de biomasa con fines energéticos. El uso de biomasa lignocelulósica como sustituto del petróleo en la producción de combustibles líquidos y de productos químicos orgánicos industriales tendría beneficios inmediatos y de gran alcance para el medio ambiente, tal como se detalla en la **Tabla 1.** La transformación de la biomasa lignocelulosa en etanol consta básicamente de tres etapas: i) pretratamiento para eliminar/modificar la estructura de la lignina y facilitar el acceso a los polisacáridos (celulosa y hemicelulosas), ii) hidrólisis enzimática (sacarificación) de los polisacáridos a azúcares simples, iii) fermentación de los azúcares simples a etanol (Zabed et al., 2017).

Tabla 1. Ventajas y desventajas del uso de biocombustibles.

VENTAJAS	DESVENTAJAS
✓ No incrementan los niveles de CO_2 en la atmósfera.	× Costo de producción casi dobla al de la gasolina
✓ Proporciona fuente de energía renovable.	× Se necesitan grandes espacios de cultivo
✓ Revitalizan economías rurales, generan empleo.	× Potenciación de monocultivos intensivos (uso de pesticidas y herbicidas)
✓ Se podrían reducir excedentes agrícolas.	× Combustible precisa de transformación previa compleja
✓ Se mejora el aprovechamiento de tierras con poco valor agrícola	× Su uso se limita a un tipo de motor de bajo rendimiento y poca potencia
✓ Se mejora la competitividad al no tener que importar fuentes de energía tradicionales	

I.3.2.1. Pretratamientos

El pretratamiento de la biomasa lignocelulósica es un requisito para una hidrólisis enzimática eficiente. Es el paso crítico, más complicado y costoso en la conversión de biomasa en etanol y puede suponer hasta un 40% del coste total del proceso (Binod et al., 2010). Los objetivos del pretratamiento son la modificación/eliminación de lignina, disminuir la cantidad de celulosa cristalina e incrementar la superficie y porosidad del material lignocelulósico para mejorar el rendimiento de sacarificación (Wyman et al., 2005). La hidrólisis de la biomasa lignocelulósica sin pretratar tiene un rendimiento del 20% de azúcares, mientras que con pretratamiento puede alcanzar hasta el 90% (Alizadeh et al., 2005). Es bien sabido que un pretratamiento puede ser más efectivo para un tipo de materia prima que para otras por lo que hay que ajustarlos a cada caso particular.

Un pretratamiento ideal debe cumplir los siguientes requisitos (Alvira et al., 2010):

1. Superar la recalcitrancia de la biomasa lignocelulósica.
2. Proporcionar un alto rendimiento en la obtención de azúcares o productos químicos y/o proporcionar un sólido pretratado altamente digerible.
3. Evitar la degradación de azúcares.
4. Evitar la formación de inhibidores y productos tóxicos.
5. Permitir la recuperación de lignina, para obtener un co-producto con valor añadido.
6. Tener buena relación coste beneficio, con tamaño de reactores razonables, poca cantidad de residuo y baja energía necesaria para llevar a cabo el proceso.

Los pretratamientos existentes se pueden clasificar en pretratamientos físicos, químicos, físico-químicos y biológicos. Las principales características de cada uno de ellos se describen a continuación.

(a) Pretratamientos físicos

En este grupo se encuentran la trituración mecánica, la pulverización por martilleo y la extrusión. Estos pretratamientos físicos pretenden producir principalmente la ruptura de la estructura lignocelulósica, reducir el tamaño de partícula y la cristalinidad de la celulosa. El objetivo final es aumentar la superficie específica y reducir el grado de polimerización.

Los pretratamientos físicos son efectivos, pero tienen como inconveniente el coste y el tiempo utilizado para reducir el tamaño de partícula, además de la energía gastada para ello, ya que supone aproximadamente un tercio de la energía total necesaria para la producción de bioetanol (Aden et al., 2002).

(b) Pretratamientos químicos

- Pretratamientos ácidos: en este pretratamiento químico, los ácidos tanto concentrados como diluidos son utilizados como catalizadores. Los ácidos concentrados permiten obtener un alto rendimiento en la obtención, a baja temperatura, de azúcares como la glucosa a partir de celulosa. El objetivo principal de los pretratamientos ácidos es solubilizar la fracción de hemicelulosas para hacer que la celulosa sea más accesible a las enzimas. Como desventajas de los ácidos concentrados encontramos la alta cantidad de ácido utilizado, la corrosión del equipo, la toxicidad del medio ambiente y la energía necesaria para recuperar el ácido (Jones y Semrau, 1984). En comparación, con los ácidos diluidos se usa menos cantidad de ácido pero son necesarias temperaturas más altas y unas condiciones de reacción más fuertes para obtener el mismo rendimiento en glucosa. Recientemente se han utilizado algunos ácidos orgánicos como los ácidos dicarboxílicos maleico, succínico, oxálico y fumárico o el ácido acético como ácido monocarboxílico (Trzcinski y Stuckey, 2015). Se cree que éstos son mejores para una biomasa con un alto contenido en celulosa y baja en hemicelulosas como, por ejemplo, las algas (Rabemanolontsoa y Saka, 2012).

- Pretratamientos alcalinos: los más comunes son los que utilizan hidróxido de sodio, potasio, calcio o amonio. La adición de un agente oxidante (oxígeno/H_2O_2) para el tratamiento previo alcalino ($NaOH/Ca(OH)_2$) puede mejorar el rendimiento al favorecer la eliminación de la lignina (Carvalheiro et al., 2008). En comparación con otros pretramientos químicos, la hidrólisis alcalina se realiza a menor temperatura y presión, provocando una menor degradación de azúcares junto con la formación de compuestos inhibidores (Carvalheiro et al., 2008), y los tiempos de reacción son de muchas horas, incluso semanas (Bali et al., 2015). Se ha demostrado que es más eficaz en residuos agrícolas que en maderas (Kumar et al., 2009).

- Ozonólisis: este pretratamiento utiliza ozono, que es un potente oxidante que muestra una alta eficacia en la deslignificación. La ozonólisis tiene como ventaja que tras la reacción no se forman compuestos inhibidores que puedan afectar a la posterior hidrólisis y fermentación. Sin embargo, tiene como inconveniente la utilización de una gran cantidad de ozono que hace inviable económicamente el proceso (Sun y Cheng, 2002).

- Organosolv: consiste en la utilización de solventes orgánicos acuosos como metanol, etanol, acetona, etilenglicol y alcohol tetrahidrofurfurílico con el objetivo de solubilizar la lignina y proporcionar celulosa adecuada para la hidrólisis enzimática (Zhao et al., 2009). Entre ellos, el etanol es el solvente más favorable porque es poco tóxico y fácil de recuperar. Como ventaja cabe destacar la recuperación de una lignina relativamente pura para su utilización como subproducto (Zhao et al., 2009). Como inconveniente encontramos el alto precio de los disolventes y los posibles efectos inhibitorios de estos sobre la hidrólisis enzimática y los microorganismos fermentativos (Sun y Cheng, 2002).

- Líquidos iónicos: los líquidos iónicos son sales orgánicas que generalmente se encuentran en estado líquido a temperatura

ambiente debido a su bajo punto de fusión. Características intrínsecas tales como alta conductividad iónica, alta potencia de solvatación, estabilidad térmica, inflamabilidad, baja volatilidad y reciclabilidad, les confieren el estatus de "disolventes verdes", dado que no forman gases tóxicos o explosivos. Estos líquidos disuelven simultáneamente la lignina y los hidratos de carbono, y como consecuencia se reduce al mínimo la formación de productos de degradación, debido a que las interacciones no covalentes entre los productos principales, celulosa, hemicelulosas y lignina, se interrumpen eficazmente. Para su aplicación a nivel industrial es indispensable el desarrollo de métodos de reciclado de estos líquidos, junto con la recuperación de hemicelulosas y lignina tras la extracción previa de celulosa (Hayes, 2009).

(c) Pretratamientos físico-químicos

- Explosión con vapor (*steam explosion*): este tratamiento es el empleado mayormente en la industria de producción de bioetanol. El proceso se lleva a cabo inyectando vapor a alta presión (20-50 bares) en un recipiente donde se introduce el sustrato a tratar, a una temperatura de 210-290 °C. La bajada de presión provoca que el vapor penetre en la estructura lignocelulósica del material que después de un tiempo de reacción determinado es expulsado hacia un ciclón a presión atmosférica. El resultado final de este tipo de pretratamiento es la alteración del empaquetamiento microfibrilar dentro de la pared celular y la rotura de la fibra, que provocan un aumento de la accesibilidad de la celulosa a las enzimas hidrolíticas. Las condiciones óptimas de temperatura y tiempo de reacción varían dependiendo del tipo de material. Como características más atractivas destaca el menor impacto ambiental, menor inversión de capital, mayor potencial de eficiencia energética, productos químicos y condiciones del proceso menos peligrosas y la recuperación completa de los carbohidratos (Avellar y Glasser, 1998). Como inconvenientes encontramos la degradación parcial de las hemicelulosas y la generación de algunos compuestos tóxicos

que pudieran afectar a las siguientes etapas de hidrólisis y fermentación (Oliva et al., 2003).

- Agua líquida caliente: al igual que los anteriores, el objetivo principal es solubilizar las hemicelulosas para hacer que la celulosa sea más accesible y para evitar la formación de inhibidores. Se realiza a temperaturas muy elevadas, cercanas a los 200 °C, y presiones elevadas para mantener el agua en estado líquido. En general, este pretratamiento tiene como ventaja que el agua actúa como solvente (no es necesario un catalizador) y medio de reacción. Además, reduce el coste en la construcción de reactores ya que la corrosión que se produce es mínima. En cuanto a los efectos sobre los materiales lignocelulósicos cabe destacar la baja concentración de hemicelulosas solubilizadas. El alto coste energético necesario para calentar el agua y la utilización de grandes cantidades de agua, hacen que este método no sea utilizado actualmente a escala industrial (Kim et al., 2016).

- Explosión de fibras con amoníaco (*ammonia fiber expansión*, AFEX): el pretratamiento con amoníaco se efectúa con cargas de amoníaco alrededor de 1:1 (kg de amoníaco/kg de biomasa seca) y a temperaturas entre 60-100 °C con tiempos de proceso muy variables dependiendo de la temperatura y alta presión durante periodos de tiempo (Alvira et al., 2010). La rápida liberación de la presión produce una ruptura física del material lignocelulósico y una reducción de la cristalinidad de la celulosa. El método AFEX elimina poca lignina y hemicelulosas pero puede mejorar la eficacia de la hidrólisis enzimática siendo necesaria una menor carga de enzimas para llevarla a cabo. Como ventaja cabe destacar que no se produce formación de inhibidores y que es un proceso respetuoso con el medio ambiente. El proceso AFEX en condiciones óptimas puede llegar a conseguir una conversión de más del 90% de celulosa y hemicelulosas en azúcares fermentables (Teymouri et al., 2005). La principal desventaja es que aunque el amoníaco puede ser

recuperado y reciclado en el reactor AFEX, el amoníaco líquido es muy caro para su aplicación industrial (Kim et al., 2016).

- Oxidación húmeda: este pretratamiento se basa en la utilización de oxígeno o aire como catalizador. El proceso consiste en una oxidación durante 10-15 minutos a temperaturas comprendidas entre 170 y 200 °C y a presiones de 10 a 12 bares de O_2. En principio, tiene como ventajas una eliminación eficaz de lignina y la formación de una baja cantidad de inhibidores.

- Pretratamiento con microondas y ultrasonido: en el primero se realiza una inmersión del material lignocelulósico en reactivos químicos diluidos, posteriormente se expone a una radiación de microondas a tiempos entre 5-20 minutos (Keshwani, 2009). El segundo se basa en la aplicación del ultrasonido sobre la biomasa Algunos autores ya han demostrado la efectividad de este pretretamiento representada en una mejora de la sacarificación de la celulosa (Yachmenev et al., 2009).

- Explosión con CO_2: es un método similar a la explosión con vapor, pero utiliza CO_2 como gas. Tiene como ventajas el bajo coste, que la toxicidad es nula al igual que la inflamabilidad del gas, fácil recuperación y un bajo impacto medioambiental (Taherzadeh y Karimi, 2008). La liberación explosiva de CO_2 a presión incrementa el área superficial de los materiales lignocelulósicos (Alvira et al., 2010). Además el CO_2 puede formar acido carbónico en presencia de humedad, el cual favorece la hidrólisis de los polímeros (Sun y Cheng, 2002).

(d) Pretratamientos biológicos

Además de los pretratamientos físicos, químicos y físico-químicos existen pretratamientos biológicos basados en el uso de hongos ligninolíticos o sus enzimas. Estos métodos utilizados en la presente Tesis se describen con más detalle en el Apartado I.4.

I.3.2.2. Hidrólisis

La producción de bioetanol a partir de material lignocelulósico se basa en la conversión de los carbohidratos celulosa y hemicelulosas en azúcares simples (glucosa, xilosa, arabinosa, galactosa,…) que posteriormente son fermentados a etanol por una serie de microorganismos. La hidrólisis puede ser llevada a cabo tanto química como enzimáticamente (Kamm et al., 2007).

Para la hidrólisis ácida se emplean los ácidos sulfúrico, clorhídrico, fluorhídrico y nítrico. La utilización de estos ácidos concentrados genera problemas, tanto en la corrosión de la maquinaria, como debido a su toxicidad (Velmurugan y Muthukumar, 2011). Debido a esto, los procesos de hidrólisis se suelen llevar a cabo con ácidos diluidos, normalmente sulfúrico o clorhídrico, a temperaturas cercanas a 200 °C. El rendimiento en la obtención de azúcares simples es limitado en la hidrólisis ácida debido a que los azúcares también se convierten en productos de degradación, como 5-hidroximetilfurfural a partir de la glucosa y furfural a partir de xilosa.

Debido al bajo rendimiento de la hidrólisis ácida, se suele usar la hidrólisis enzimática con celulasas que catalizan la hidrólisis de la celulosa en glucosa y es 100% selectiva. Estas enzimas son producidas por varios microorganismos, pero el más común es el hongo *Trichoderma reesei*, aunque también el hongo *Aspergillus niger* y la bacteria *Clostridium cellulovorans* las producen (Arai et al., 2006).

Debido a las características estructurales de la celulosa, no podemos obtener de manera directa glucosa por la acción de una celulasa. Es necesaria la acción de endo- y exo-glucanasas y β-glucosidasas, enzimas que actúan sobre la celulosa de manera simultánea y sinérgica. Las endoglucanasas rompen fragmentos de celulosa desde el interior, mientras que la exoglucanasas lo hacen desde los extremos de la cadena; con la acción de ambas enzimas se forman moléculas de celobiosa que son hidrolizadas por la β-glucosidasa en moléculas simples de glucosa.

I.3.2.3. Fermentación

La fermentación es un proceso que convierte los monosacáridos (hexosas y pentosas) en etanol mediante el uso de microorganismos. El microorganismo ideal debe producir un alto rendimiento en la producción de etanol, tolerar altas concentraciones de éste y de los inhibidores producidos durante el proceso y tener la capacidad de fermentar azúcares a altas temperaturas (Arai et al., 2006). Los organismos más usadas son las levaduras, como *Saccharomyces cerevisiae* y *Candida utilis*, y bacterias, como *Zymomona mobilis*; estos microorganismos poseen alta tolerancia al etanol y altos rendimientos de producción, entre un 90-97%.

I.4. BIODEGRADACIÓN ENZIMÁTICA DE LA LIGNINA

Como se describió anteriormente, la producción de bioetanol a partir de material lignocelulósico requiere la deconstrucción de la pared celular en polímeros individuales, y la hidrólisis de los carbohidratos en azúcares. La cantidad de lignina, así como su composición y estructura son uno de los principales factores que causan la recalcitrancia de la biomasa (Studer et al., 2011).

La lignina es un polímero tridimensional constituido por subunidades de fenilpropano unidas entre sí por una variedad de enlaces éter y carbono-carbono. La lignina está íntimamente entrelazada con hemicelulosas en la pared celular de la planta formando una matriz que cubre las microfibrillas de celulosa cristalina. Su naturaleza aromática y estructura compleja hace que la degradación de la lignina sea muy difícil. La lignina dificulta el acceso a las celulasas y también se une a ellas produciendo su inactivación (Rahikainen et al., 2013). Como se ha descrito anteriormente, el pretratamiento de la biomasa para eliminar o modificar la lignina es esencial para mejorar la hidrólisis enzimática de los polisacáridos. Además de los pretratamientos físicos o químicos, también se están desarrollando pretratamientos biológicos para deconstruir la biomasa lignocelulósica y eliminar la lignina (Alvira et al., 2010). La mayoría de los pretratamientos biológicos emplean hongos que degradan la lignina y que pertenecen al grupo de basidiomicetos de podredumbre blanca, pero tales tratamientos

previos requieren largos períodos de aplicación y consumen una fracción de los polisacáridos de la planta, reduciendo el rendimiento. Sin embargo, el uso de enzimas ligninolíticas procedentes de estos hongos puede contribuir a la deconstrucción de biomasa vegetal al proporcionar nuevos biocatalizadores capaces de degradar o modificar la lignina.

En la degradación natural de la pared celular vegetal hay varios tipos de enzimas involucradas. Este es un proceso secuencial en el que la eliminación de lignina es a menudo el primer paso y el que limita la velocidad. Una vez que se elimina la protección conferida por la lignina recalcitrante, los polisacáridos de la pared celular son susceptibles al ataque de las enzimas hidrolíticas. Las únicas enzimas conocidas capaces de degradar la lignina son las utilizadas por los basidiomicetos ligninolíticos, los llamados hongos de podredumbre blanca debido al color blanquecino del sustrato enriquecido con celulosa después de la eliminación de la lignina. Estos organismos han desarrollado un mecanismo basado en oxidorreductasas de alto potencial redox. Estas enzimas incluyen lacasas (fenoloxidasas fúngicas) y peroxidasas ligninolíticas, como la lignina peroxidasa (LiP), la manganeso peroxidasa (MnP) o la peroxidasa versátil (VP), que actúan en sinergia con oxidasas productoras de H_2O_2, entre otras enzimas. La degradación de la lignina por las oxidorreductasas anteriores se definió como una "combustión enzimática" (Kirk y Farrell, 1987) debido al ataque enzimático inespecífico, que está dirigido hacia el anillo bencénico de las estructuras de la lignina. Debido a la naturaleza voluminosa de la lignina, las peroxidasas que degradan la lignina han desarrollado un mecanismo digno de mención que permite la oxidación del polímero en la superficie de la enzima por un radical proteico expuesto, y posterior transferencia de electrones de largo alcance al cofactor del hemo activado por peróxido (Ruiz- Dueñas y Martínez, 2009).

Las lacasas (fenoloxidasas, EC 1.10.3.2) son oxidasas multicobre que oxidan fenoles sustituidos utilizando oxígeno molecular como el aceptor final de electrones (**Figura 17**). La acción directa de las lacasas sobre la lignina está, en principio, restringida a unidades fenólicas que sólo representan un pequeño porcentaje del polímero total, un hecho que

limita su aplicación biotecnológica (Riva, 2006). Una alternativa es el uso de mediadores redox, compuestos simples que forman radicales difusibles estables, que una vez oxidados por la enzima, actúan a distancia e incluso cuando el acceso al polímero de lignina está limitado por impedimentos estéricos (como la estructura compacta de la pared celular de la planta en materiales no deteriorados). El descubrimiento de algunos compuestos sintéticos que pueden actuar como transportadores de electrones entre la enzima y el sustrato final, como es el caso del 1-hidroxibenzotriazol (HBT), ha ampliado la utilidad de las lacasas. Varios estudios han confirmado el potencial de los sistemas mediadores de lacasa para la deslignificación de pasta de papel (Babot et al., 2011), el control del pitch (Gutiérrez et al., 2009), y otras aplicaciones en la industria forestal (Widsten y Kandelbauer, 2008) así como en la producción de bioetanol a partir de lignocelulosa pretratada física y/o químicamente (Palonen y Viikari, 2004b).

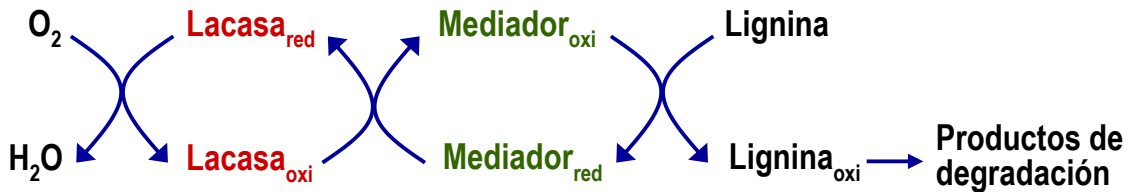


Figura 17. Mecanismo de actuación del sistema lacasa-mediador sobre la lignina.

La capacidad de lacasas de alto potencial redox aisladas de los basidiomicetos *Trametes villosa* o *Picnoporus cinnabarinus* (**Figura 18**), en presencia de HBT como mediador, para eliminar la lignina tanto de plantas madereras (eucalipto) como de plantas anuales (hierba elefante), haciendo que la celulosa sea más accesible a la hidrólisis enzimática, ha sido recientemente demostrada (Gutiérrez et al., 2012; Rico et al., 2014a,b). Posteriormente, también se demostró la capacidad de una lacasa de bajo potencial redox, aislada del ascomiceto *Myceliophthora thermophila*, en presencia de mediadores fenólicos, para eliminar/modificar la lignina de madera de eucalipto y mejorar la sacarificación enzimática (Rico et al., 2014a).

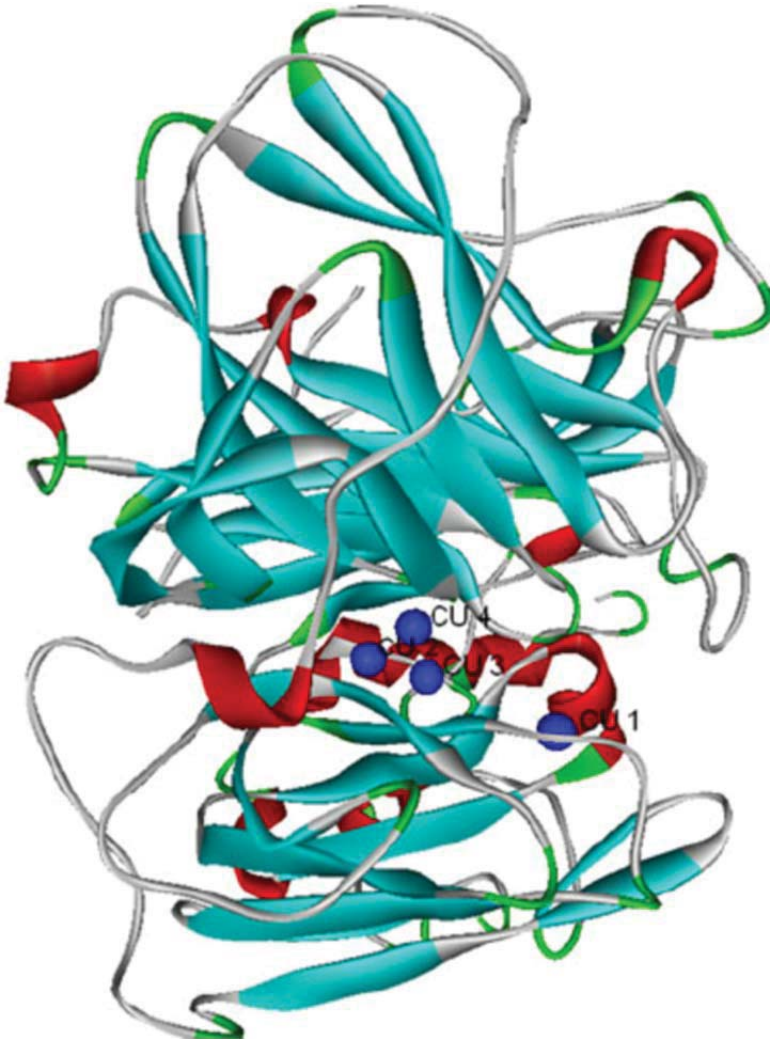


Figura 18. Representación tridimensional de la estructura molecular de la lacasa del hongo *Pycnoporus cinnabarinus* mostrando las regiones en hélice α (rojo), hoja plegada β (cian), los giros β (verde) y los átomos de cobre catalíticos en color azul (Prasad et al., 2011).

I.5. INFLUENCIA DE LA LIGNINA EN LA SACARIFICACIÓN DEL MATERIAL LIGNOCELULÓSICO

El contenido en lignina es uno de los factores limitantes en la digestibilidad enzimática de la celulosa (Ding, Liu et al. 2012). La presencia de lignina residual tras los pretratamientos convencionales produce un efecto negativo en la producción de etanol debido a los siguientes mecanismos:

- La lignina puede unirse a las celulasas empleadas para la sacarificación, de manera no específica (bloqueando los sitios activos e impidiendo la unión a la celulosa), dando lugar a la reducción de la actividad catalítica de estas enzimas (Mansfield et al., 1999; Eriksson et al., 2002; Moilanen et al., 2011).

- La lignina que permanece unida a la celulosa tras los pretratamientos reduce el área superficial disponible para la hidrólisis enzimática de la celulosa (Moilanen et al., 2011).

- Los productos derivados de la lignina como los fenoles aromáticos ácidos y aldehídos, son a menudo tóxicos para las levaduras que llevan a cabo la fermentación. Se ha descubierto que pequeñas concentraciones de estos inhibidores destruyen la integridad del sistema de membrana de la levadura, impidiendo el crecimiento de ésta (Palmqvist et al., 1999; Palmqvist y Hahn-Hägerdal, 2000; Himmel et al., 2007).

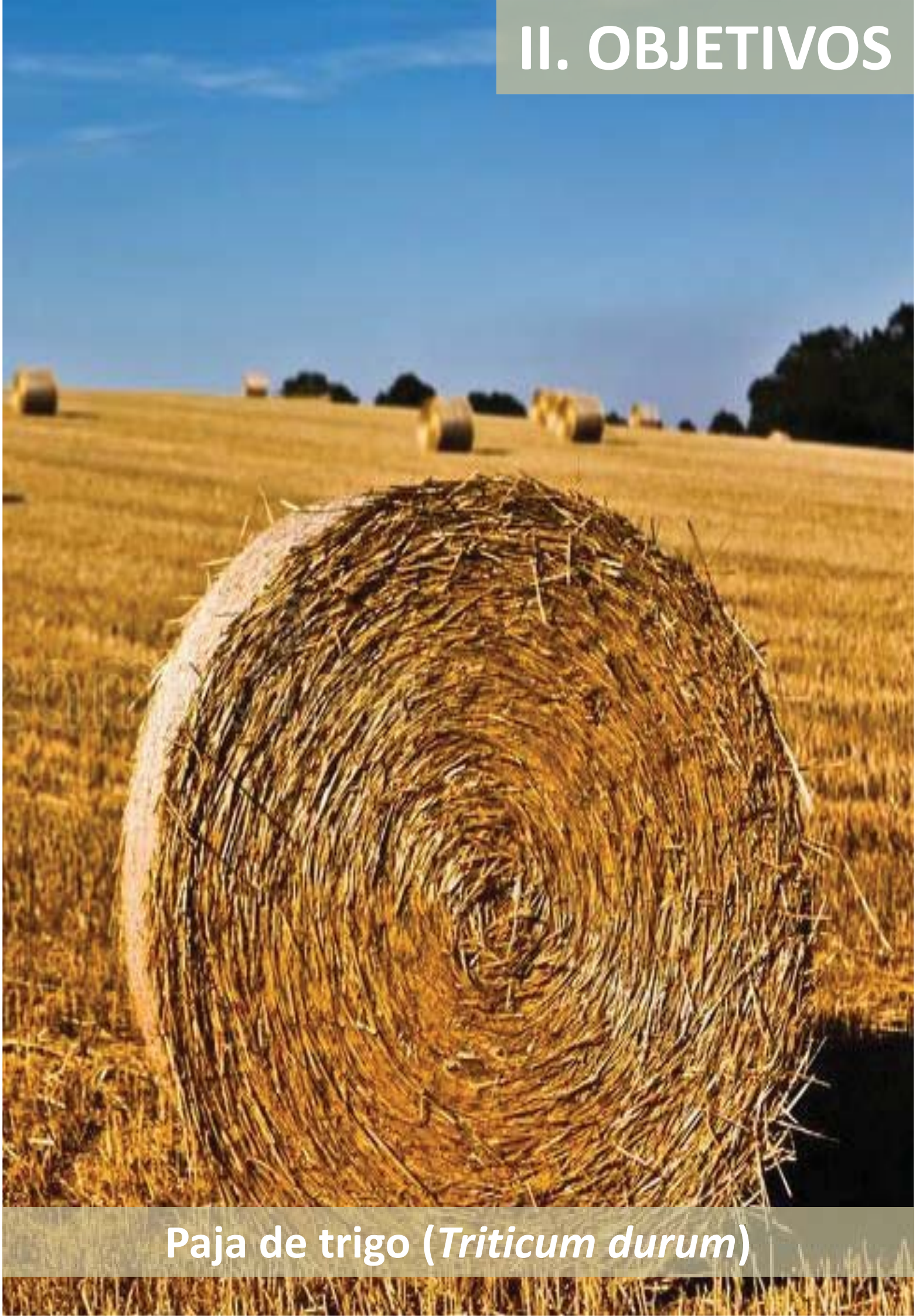
Durante la hidrólisis enzimática de material lignocelulósico pretratado, las celulasas se unen a la superficie de la lignina (Palonen et al., 2004). Esta unión se considera perjudicial tanto en el proceso de hidrólisis de celulosa por las enzimas, como en la recuperación enzimática. Las interacciones lignina-celulasa están siendo estudiadas, sin embargo la razón fundamental que explica el efecto negativo de la lignina en la hidrólisis enzimática aún no se sabe. Se han realizado estudios del efecto inhibitorio de la lignina pretratada con steam explosion (Rahikainen et al., 2013).

En varios estudios (Nakagame et al., 2010; Nakagame et al., 2011), se ha observado que la lignina aislada de especies madereras muestra una mayor inhibición en la hidrólisis enzimática de celulosa Avicel que la lignina extraída de una planta herbácea (ej. maíz). Esto sugiere que la diferente composición en la lignina nativa también puede influir en la inhibición de las celulasas.

En la presente Tesis se estudió el sistema lacasa-mediador como pretratamiento para la deslignificación de materiales lignocelulósicos con

el fin de mejorar la posterior sacarificación enzimática con celulasas. A su vez se estudió el papel de la lignina en la inhibición enzimática por la adsorción no productiva de celulasas.

II. OBJETIVOS



Paja de trigo (*Triticum durum*)

La presente Tesis aborda el estudio y desarrollo de pretratamientos enzimáticos sobre diferentes materiales lignocelulósicos con el objetivo de conseguir un mejor aprovechamiento industrial de los mismos, en particular la obtención de bioetanol de segunda generación. Se enfoca especialmente en el estudio de la lignina, tanto en su eliminación/modificación durante los pretratamientos enzimáticos, como en su acción relacionada con la inhibición de las celulasas utilizadas para la sacarificación de la celulosa.

Los objetivos específicos de la Tesis son los siguientes:

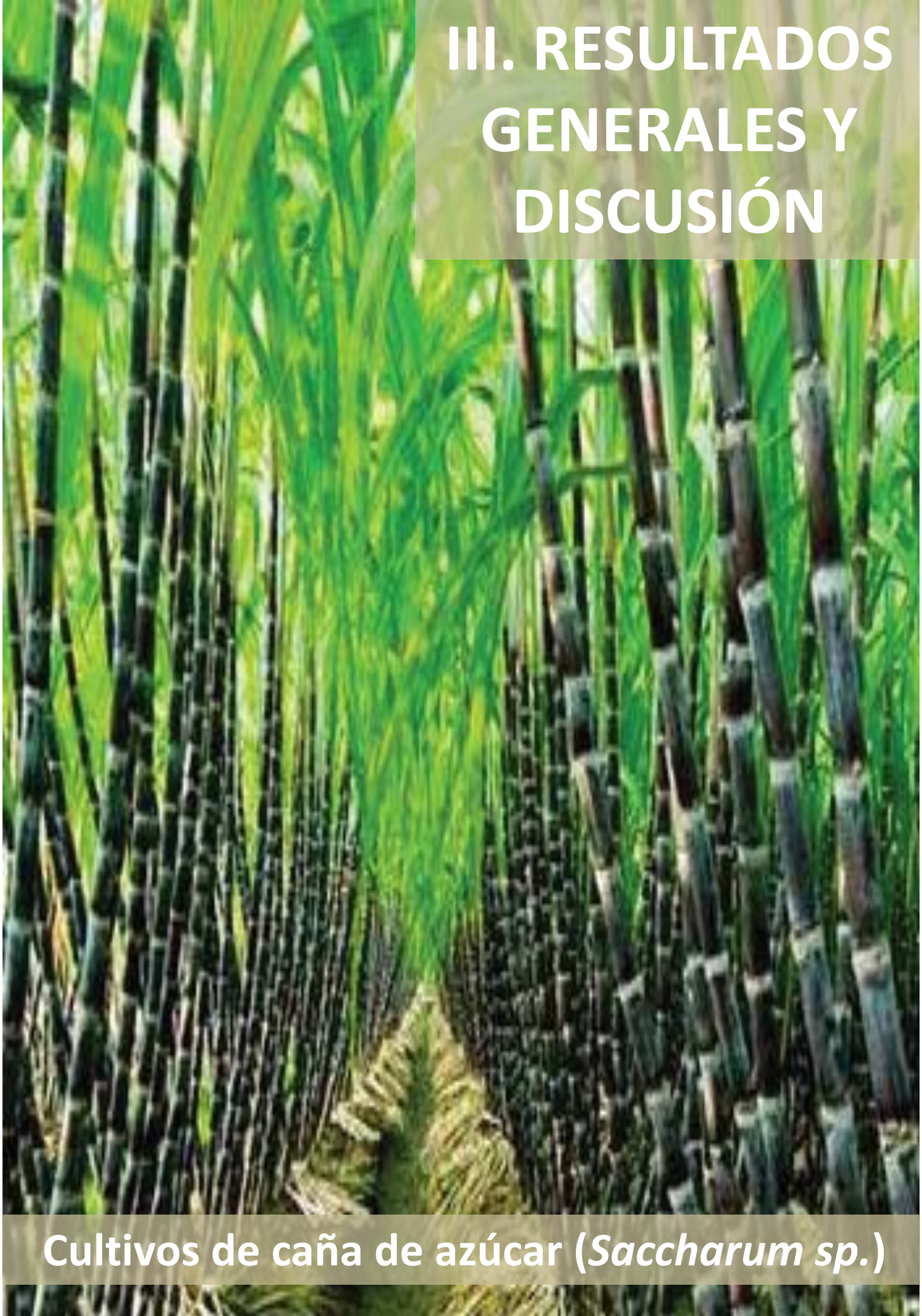
1. Desarrollar pretratamientos enzimáticos basados en el sistema lacasa-mediador con el fin de degradar la lignina presente en los materiales lignocelulósicos procedentes de residuos agrícolas (paja de trigo y bagazo y paja de caña de azúcar) y de cultivos forestales de crecimiento rápido (*Paulownia fortunei*), con el fin de mejorar el rendimiento de la sacarificación enzimática para la obtención de bioetanol de segunda generación.

2. Analizar y estudiar en detalle mediante Resonancia Magnética Nuclear bidimensional (2D-NMR) las modificaciones estructurales que tienen lugar en la lignina durante dichos pretratamientos.

3. Evaluar la mejora en la sacarificación enzimática con celulasas de los distintos materiales lignocelulósicos pretratados enzimáticamente.

4. Estudiar las interacciones que se producen entre las celulasas y la lignina aislada de diferentes materias primas, tanto madereras (eucalipto y pícea) como no madereras (paja de trigo).

III. RESULTADOS GENERALES Y DISCUSIÓN



Cultivos de caña de azúcar (*Saccharum sp.*)

En la presente Tesis se ha estudiado, por un lado, la efectividad del sistema lacasa-mediador como pretratamiento enzimático para deslignificar y mejorar la sacarificación de diversos materiales lignocelulósicos con vistas a la obtención de bioetanol de segunda generación, y por otro lado las interacciones entre la lignina y las celulasas usadas para la hidrólisis enzimática, que conlleva una reducción de su actividad catalítica.

III.1. PRETRATAMIENTOS ENZIMÁTICOS CON EL SISTEMA LACASA-MEDIADOR

Se estudió el efecto de pretratamientos enzimáticos basados en el sistema lacasa-mediador sobre diferentes materiales lignocelulósicos de interés industrial, con el fin de eliminar y/o modificar la lignina presente en las materias primas para obtener una mayor accesibilidad de las celulasas a la celulosa con vistas a mejorar la sacarificación y el rendimiento en bioetanol celulósico. A su vez, se analizaron en detalle las modificaciones estructurales que sufre el polímero de lignina durante dichos pretratamientos mediante Resonancia Magnética Nuclear bidimensional (2D-NMR) para conocer el mecanismo de degradación.

Estos estudios se realizaron sobre diferentes materiales lignocelulósicos tales como la paja de trigo y el bagazo y la paja de caña de azúcar, residuos abundantes de la actividad agraria, así como sobre madera de paulownia, una especie de crecimiento rápido.

III.1.1. Pretratamiento enzimático de paja de trigo con lacasa de *Pycnoporus cinnabarinus* y HBT

Los pretratamientos enzimáticos sobre paja de trigo se realizaron con el sistema lacasa-mediador, seguido de una extracción alcalina. Para ello, se utilizó la lacasa de *P. cinnabarinus* a diferentes dosis (13 y 65 $U \cdot g^{-1}$) y como mediador se utilizó HBT en diferentes concentraciones; 5, 10 y 20%.

Los resultados obtenidos demostraron la eficacia del pretratamiento enzimático en la modificación y eliminación de lignina de paja de trigo. El

mejor resultado se obtuvo cuando se utilizó 65 $U \cdot g^{-1}$ de lacasa y HBT al 20%, seguido de una extracción alcalina posterior, produciéndose una reducción del 45% en el contenido de lignina respecto a la materia prima sin pretratar. El pretratamiento utilizando enzima sola (65 $U \cdot g^{-1}$) sin mediador consiguió una reducción del contenido en lignina del 18%, demostrando la eficacia de la enzima aisladamente, aunque en menor medida. Por otro lado, el pretratamiento con el sistema lacasa-mediador en las condiciones mencionadas anteriormente, pero sin extracción alcalina posterior, produjo un descenso del 37% del contenido de lignina, indicando la buena eficacia de este sistema enzimático para reducir el contenido de lignina en paja de trigo.

Estos estudios también evidenciaron que la eliminación de lignina en la paja de trigo está directamente relacionada con un incremento en la obtención de glucosa tras la hidrólisis enzimática (sacarificación). Para la hidrólisis de los polisacáridos se usó un cóctel enzimático de la empresa Novozymes, que contiene exo- y endocelulasas (Celluclast 2 FPU$\cdot g^{-1}$) y β-glucosidasa (6 $U \cdot g^{-1}$). Se observó una mejora del 60% en la liberación de glucosa en la sacarificación de la paja de trigo pretratada con el sistema lacasa-mediador a dosis altas (65 $U \cdot g^{-1}$ lacasa y HBT al 20%), como se indica la **Tabla 2.**

Estudios anteriores indicaron que la lignina de la paja de trigo utilizada en estos tratamientos se caracteriza por presentar unidades *p*-hidroxifenilo (H), guayacilo (G) y siringilo (S), estando enriquecida en unidades G (H:G:S, 6:64:30), con un claro predominio de unidades G. Además, la lignina de la paja de trigo también incorpora importantes cantidades de tricina, la cual actúa como un verdadero monómero, así como ácidos *p*-cumárico y ferúlico (del Río et al., 2012). Las modificaciones estructurales producidas en el polímero de lignina durante el pretratamiento enzimático se estudiaron en detalle mediante Resonancia Magnética Nuclear bidimensional (2D-RMN). En la **Figura 19** se muestran los espectros de HSQC 2D-NMR de la paja de trigo tratada con el sistema lacasa-mediador (65 $U.g^{-1}$ de lacasa y HBT 20%), y tras ser sometida a una extracción alcalina posterior, y los espectros correspondientes a la paja de trigo control.

Tabla 2. Contenido en lignina (determinada como lignina klason) y porcentaje de glucosa liberada tras la hidrólisis enzimática de las muestras de paja de trigo pretratadas por el sistema lacasa-mediador (*P. Cinnabarinus* y HBT), así como de la materia prima inicial.

Muestras de paja de trigo	Lignina (%)	Glucosa (%)
Materia prima inicial	16.0 ± 0.3	21.6 ± 0.2
-Lacasa-mediador		
Control	15.6 ± 0.2	24.1 ± 0.0
Lacasa (65 $U \cdot g^{-1}$)	15.0 ± 0.1	29.0 ± 0.1
Lacasa (65 $U \cdot g^{-1}$); HBT (20%)	9.8 ± 0.3	33.6 ± 0.2
-Lacasa-mediador + extracción alcalina		
Control	14.8 ± 0.2	28.4 ± 0.4
Lacasa (65 $U \cdot g^{-1}$)	12.1 ± 0.3	31.1 ± 0.3
Lacasa (13 $U \cdot g^{-1}$); HBT (10%)	9.4 ± 0.1	35.1 ± 0.5
Lacasa (65 $U \cdot g^{-1}$); HBT (5%)	9.2 ± 0.4	36.5 ± 0.3
Lacasa (65 $U \cdot g^{-1}$); HBT (20%)	7.7 ± 0.2	41.5 ± 0.7
Lacasa (65 $U \cdot g^{-1}$); HBT (20%)/T20	7.5 ± 0.2	45.5 ± 0.6

El análisis de las regiones aromáticas de los espectros HSQC reveló que el tratamiento con el sistema lacasa-mediador produce una eliminación de las unidades H, G y S de lignina, además de los ácidos *p*-cumárico y ferúlico, y un aumento paralelo de las unidades de lignina oxidadas (G' y S'). Cabe destacar la resistencia de la tricina al pretratamiento enzimático, aunque se observa un ligero descenso de esta. Por otro lado, el análisis de las regiónes alifáticas de los espectros HSQC mostró un descenso en las señales correspondientes a los enlaces de lignina (β-*O*-4´, β-β´y β-5´), indicando la despolimerización de la lignina, mientras que las señales de carbohidratos (X: xilosa y Gl: glucosa) no se vieron afectadas por el tratamiento. En conclusión, la ruptura de los enlaces dentro del polímero de lignina y el aumento de las unidades de lignina oxidada ponen de manifiesto que el sistema lacasa-mediador sigue un mecanismo de despolimerización oxidativa.

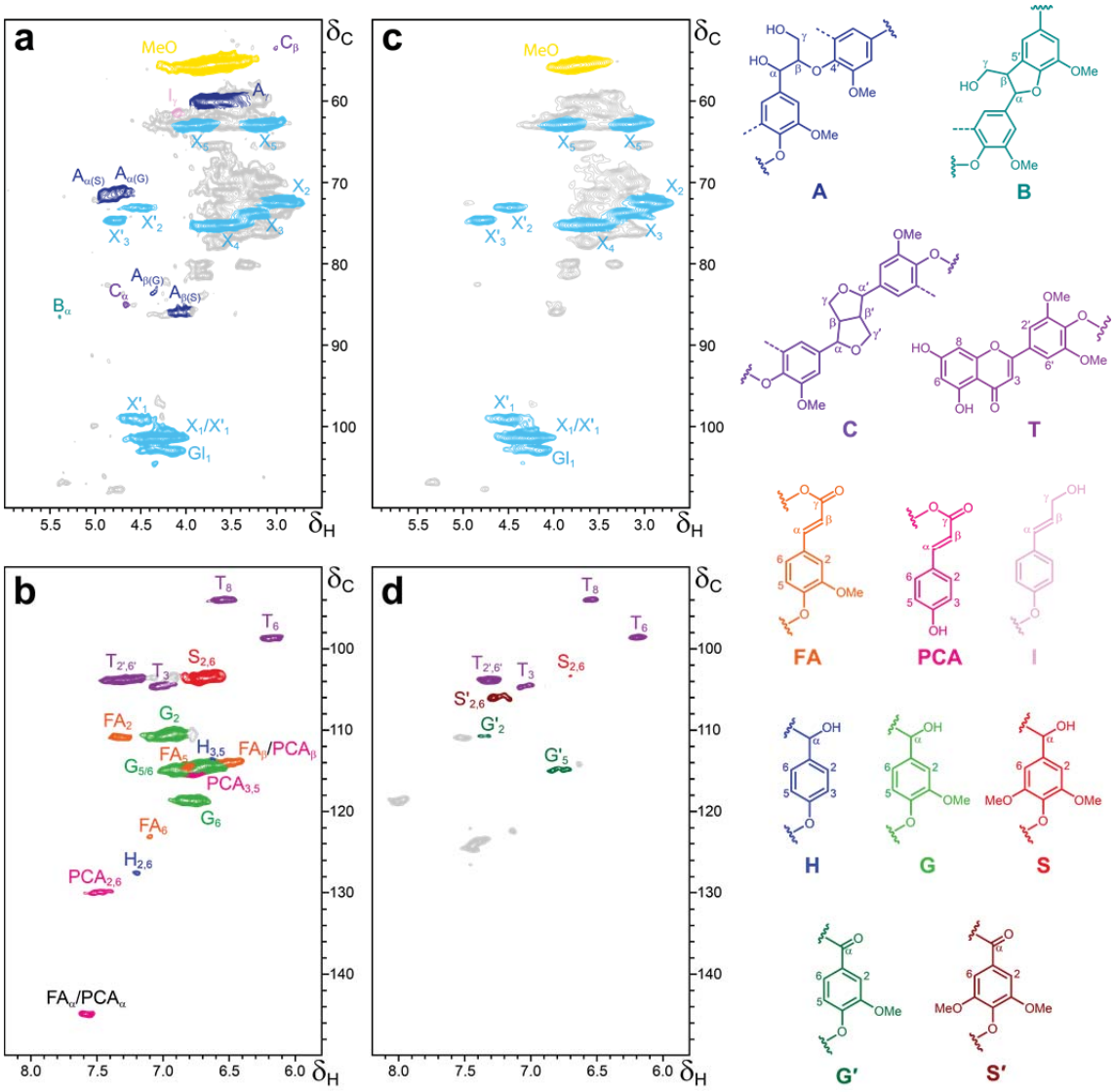


Figura 19. Regiónes alifáticas (a y c) y aromáticas (b y d) de los espectros HSQC 2D-NMR de las muestras de paja de trigo tratada con el sistema lacasa-mediador (c y d), utilizando 65 U·g^{-1} de lacasa y HBT 20%, y de su correspondiente control (sin enzima ni mediador). Principales estructuras identificadas en los espectros HSQC: β-*O*-4' alquil aril éter (A), β-5' fenilcumarano (B), β-β' resinol (C), tricina (T), ácido *p*-cumárico (PCA), ácido ferúlico (FA), alcohol cinamílico terminal, unidades *p*-hidroxifenilo (H), unidades guayacilo (G), unidades siringilo (S), unidades G oxidadas (G') y unidades S oxidadas (S') .

III.1.2. Pretratamiento enzimático de bagazo y paja de caña de azúcar con lacasa de *Pycnoporus cinnabarinus* y HBT

El mismo sistema lacasa-mediador utilizado con la paja de trigo fue evaluado sobre el bagazo y la paja de la caña de azúcar, cuyas ligninas presentan una composición y estructura muy diferentes. Mientras que la lignina del bagazo de caña de azúcar contiene predominantemente

unidades de tipo S y enlaces β-éter no condensados, la lignina de la paja está enriquecida en unidades de tipo G y presenta enlaces más condensados (del Río et al., 2015). Este estudio nos permitió conocer el efecto de la composición de la lignina sobre la eficiencia del pretratamiento con el sistema lacasa-mediador.

El pretratamiento con lacasa de *P. cinnabarinus* y HBT sobre los residuos de la caña de azúcar no fue tan efectivo en la eliminación de lignina como en el caso de la paja de trigo. Por ello, alternativamente, se evaluó un pretratamiento que consistió en usar menos dosis de enzima y mediador, pero repitiendo el tratamiento en cuatro ciclos. Se utilizaron 50 $U \cdot g^{-1}$ de lacasa y HBT al 3% en cada uno de los 4 ciclos. Bajo estas condiciones se consiguió un descenso del 27% y 31% en el contenido de lignina en el bagazo y la paja de caña de azúcar, respectivamente **(Tabla 3)**.

Tabla 3. Contenido en lignina y porcentaje de glucosa liberada tras la hidrólisis enzimática de las muestras de paja y bagazo de caña azúcar tratadas con el sistema lacasa-mediador (*P. cinnabarinus* y HBT), así como de las materias primas iniciales.

Muestras	Lignina (%)	Glucosa (%)
Materia prima bagazo caña de azúcar	17.8 ± 0.6	35.9 ± 0.7
-LMS + extracción alcalina (4 ciclos)		
Control	17.5 ± 0.4	40.1 ± 0.2
Lacasa (50 $U \cdot g^{-1}$)	16.8 ± 0.3	44.2 ± 0.4
Lacasa (50 $U \cdot g^{-1}$); HBT (3%)	12.8 ± 0.3	55.8 ± 0.4
Materia prima paja caña de azúcar	17.0 ± 0.2	34.9 ± 0.2
-LMS + extracción alcalina (4 ciclos)		
Control	16.6 ± 0.2	39.2 ± 0.2
Lacasa (50 $U \cdot g^{-1}$)	15.1 ± 0.1	42.3 ± 0.1
Lacasa (50 $U \cdot g^{-1}$); HBT (3%)	11.5 ± 0.3	57.1 ± 0.3

También se observó que el tratamiento con lacasa sola, en ausencia de HBT, resultó mucho menos efectivo, observándose tan solo un descenso del 4% y 9% en el contenido en lignina, en bagazo y paja, respectivamente. Al igual que ocurrió tras el pretratamiento de la paja de trigo, el descenso

en el contenido de lignina de los residuos de la caña de azúcar llevó también asociado un incremento en la obtención de glucosa tras la hidrólisis enzimática. En este caso, se consiguió una mejora del 39% y 46% en el rendimiento de glucosa para el bagazo y paja de caña de azúcar pre-tratadas con el sistema lacasa-mediador, en comparación con el control.

Por otro lado, se estudió en detalle las modificaciones estructurales de la lignina durante los tratamientos enzimáticos mediante 2D-NMR (**Figura 20**). Tanto en el bagazo como en la paja de la caña de azúcar, se observó un descenso significativo en los enlaces y cantidad de lignina, confirmándose el mecanismo de despolimerización oxidativa de la lignina.

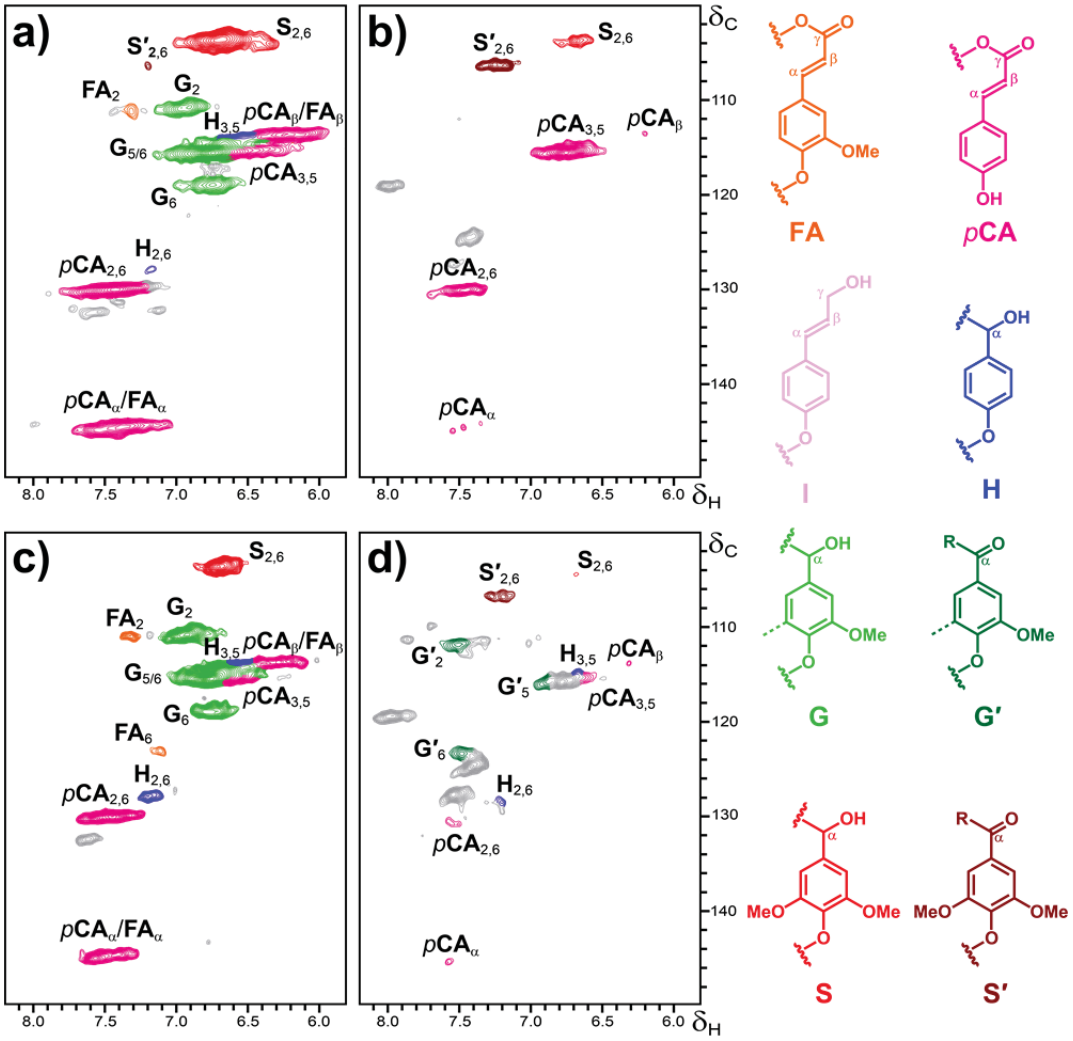


Figura 20. Regiones aromáticas de los espectros HSQC de la paja (arriba) y el bagazo (abajo) de la caña de azúcar, junto con las principales estructuras de lignina identificadas en los mismos. Los espectros a) y c) corresponden a los controles mientras que los espectros b) y d) corresponden a las muestras tratadas.

III.1.3. Pretratamiento enzimático de *Paulownia fortunei* con lacasa de *Myceliophthora thermophila* y siringato de metilo

El tercer pretratamiento con el sistema lacasa-mediador estudiado en la presente Tesis utilizó como sustrato la madera de paulownia (*P. fortunei*), una especie de crecimiento rápido cuya lignina está compuesta principalmente por unidades de tipo G (60%) y S (40%) (Rencoret et al., 2009).

En este estudio, el pretratamiento consistió en 4 ciclos, donde cada uno de los ciclos incluía el tratamiento con el sistema lacasa-mediador seguido de una extracción alcalina con peróxido de hidrógeno. A diferencia de los dos anteriores, el sistema lacasa mediador en este caso estaba compuesto por la lacasa comercial del hongo *M. thermophila* (MtL) y siringato de metilo (MeS) como mediador. En cada uno de los ciclos se utilizaron 50 $U \cdot g^{-1}$ de MtL y MeS al 3%. Bajo estas condiciones, se consiguió un descenso de tan solo un 24% del contenido de lignina de paulownia, una reducción mucho menor que la que se consiguió cuando se trató madera de eucalipto (50% de reducción), utilizándose las mismas condiciones (Rico et al., 2014). Esta diferencia puede ser debida a la diferente composición de la lignina en ambos sustratos, ya que en la madera de eucalipto predominan las unidades de tipo S (relación S/G de 3.6) y los enlaces β-éter no condensados (más fáciles de degradar), mientras que la lignina de paulownia contiene predominantemente unidades de tipo G (relación S/G de 0.8) y presenta una estructura con más enlaces C-C y, por tanto, más condensada.

Al igual que ocurrió con otras materia primas pretratadas, el descenso en el contenido de lignina estuvo acompañado de un incremento en la obtención de glucosa tras la hidrólisis enzimática. Utilizando el mismo cóctel enzimático que en los casos anteriores, se obtuvo una mejora del 40% en la liberación de glucosa (**Tabla 4**).

Tabla 4. Contenido en lignina (determinada como lignina klason) y porcentaje de glucosa liberada tras la hidrólisis enzimática de las muestras de paulownia tratadas con el sistema lacasa-mediador (*M. thermophila* y MeS), así como de la muestra de paulownia inicial.

Muestras de *Paulownia*	**Lignina (%)**	**Glucosa (%)**
Materia prima	23.8 ± 0.2	31.5 ± 0.3
Control	22.0 ± 0.1	37.3 ± 0.2
Lacasa (50 U g^{-1})	20.9 ± 0.3	39.4 ± 0.4
Lacasa (50 U g^{-1}) MeS (3%)	16.7 ± 0.2	51.3 ± 0.4

Al igual que en los pretratamientoe anteriores, las cambios estructurales producidos en la lignina de la madera de paulownia, como consecuencia del tratamiento con el sistema lacasa-mediador, se estudiaron por 2D-NMR (**Figura 21**). Los espectros HSQC 2D-NMR mostraron una pronunciada eliminación de las señales de lignina (en color rojo) después del pretratamiento enzimático con lacasa y MeS, en comparación con la muestra control, aunque también se observó cierta eliminación de lignina en el espectro corresponsiente a la muestra de paulownia tratada con lacasa sola. Por su parte las señales correspondientes a los carbohidratos (azul) permanecieron prácticamente invariables.

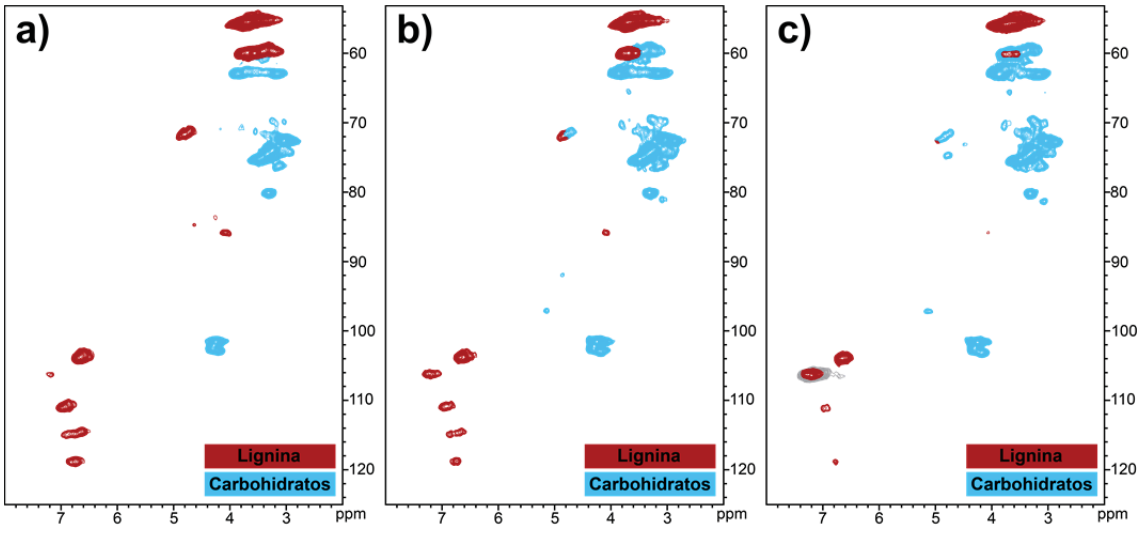


Figura 21. Espectros HSQC 2D-NMR de las maderas de paulownia tratadas con el sistema lacasa-mediador y extraídas posteriormente con peróxido alcalino (4 ciclos): (a) control sin enzima, (b) paulownia tratada con lacasa (50 U·g^{-1}) y (c) tratado con lacasa de *M. thermophila* (50 U·g-1) y MeS (3%). Las señales de correlación en color rojo corresponden a lignina, mientras que las señales en azul pertenecen a los carbohidratos.

El análisis detallado de las regiónes aromáticas de los espectros HSQC (**Figura 22**) mostró una disminución considerable de las señales correspondientes a las unidades G y S de la lignina, con respecto al control, tras el tratamiento enzimático. Además, se observó un aumento significativo en la intensidad de las señales correspondientes a unidades de lignina oxidadas en su posición alfa (G′ y S′), como se evidencia por el aumento relativo en las señales $S'_{2,6}$ y la aparición de las señales G'_2, G'_5 y G'_6, confirmándose una vez más el carácter oxidativo de la despolimerización enzimática. En el tratamiento con lacasa sola, sin mediador, también se pudo observar, aunque en menor medida, una oxidación de las unidades del polímero de lignina.

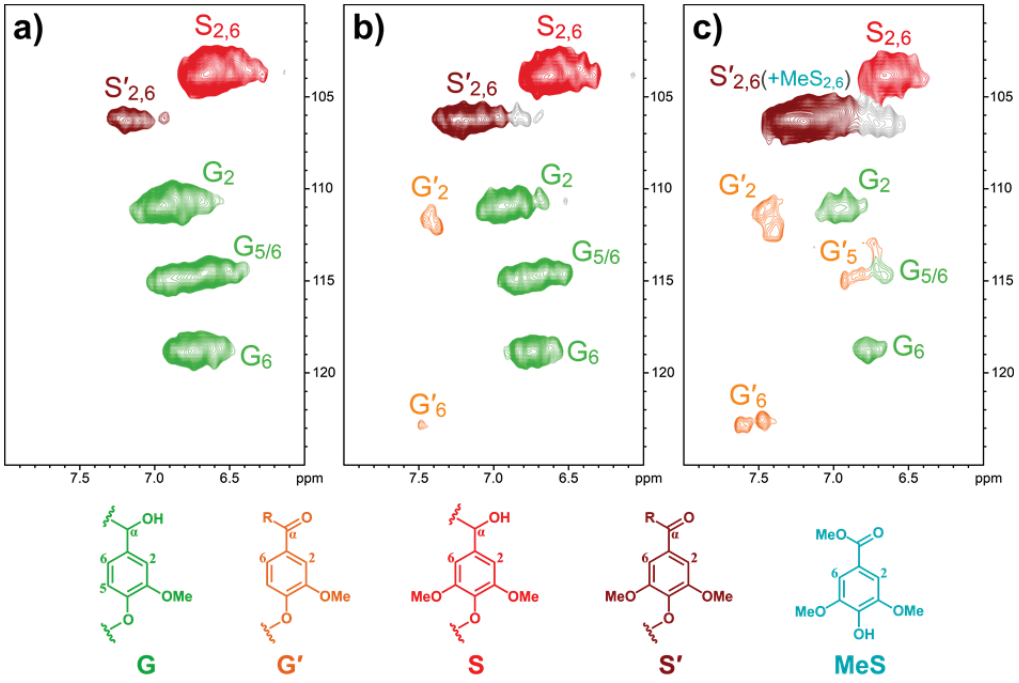


Figura 22. Regiónes aromáticas (δ_C/δ_H 100-125/6.0-8.0) de los espectros HSQC de las muestras de paulownia tratadas con el sistema lacasa-mediador; (a) Control sin enzima, (b) tratada con enzima sola 50 $U \cdot g^{-1}$ y (c) tratada con 50 $U \cdot g^{-1}$ de enzima y 3% de MeS.

Por último, se analizaron los filtrados de los tratamientos para confirmar las reacciones de despolimerización que tienen lugar durante el pretratamiento de la madera de paulownia. Las fracciones solubles en cloroformo de los filtrados se analizaron mediante GC-MS para determinar

la presencia de compuestos de bajo peso molecular derivados de lignina (**Figura 23**).

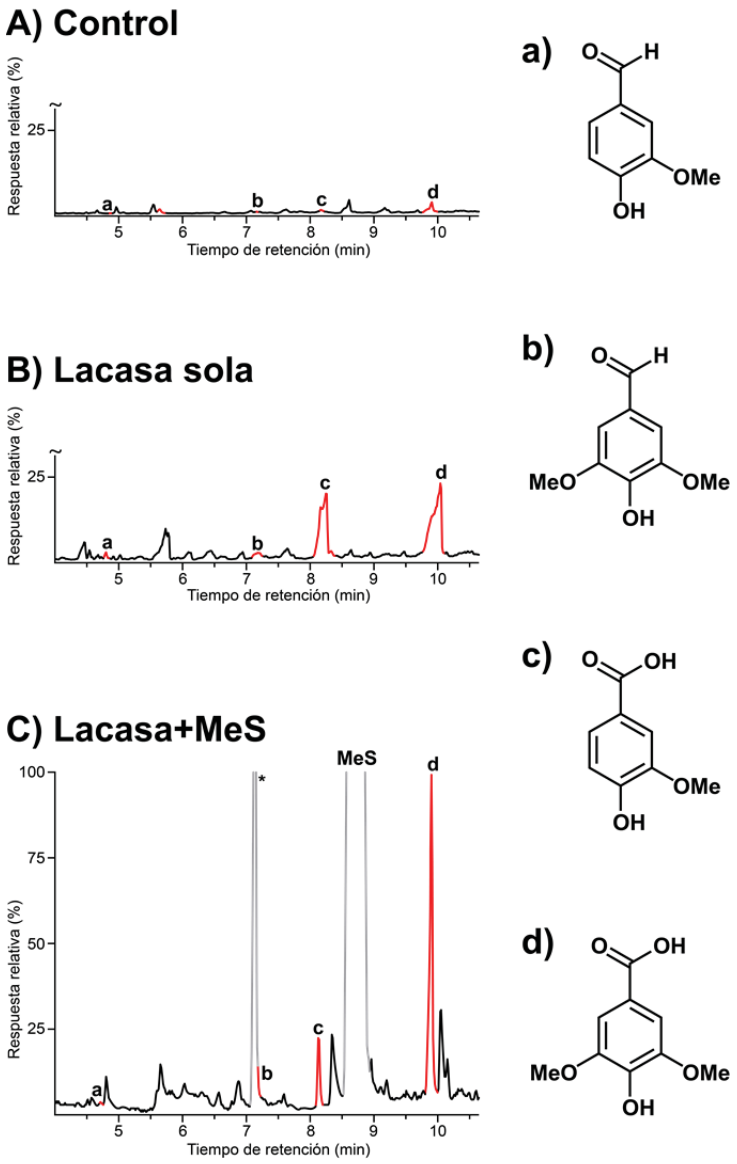


Figura 23. Cromatogramas de la fracción soluble en cloroformo de los filtrados obtenidos después del pretratamiento enzimático de la madera paulownia con sistema lacasa-mediador (C), lacasa sola (B) y el control correspondiente (A). Estructura de los compuestos simples derivados de lignina identificados (a-e). El pico con marca de asterisco (*) corresponde a una contaminación del siringato de metilo (MeS) utilizado como mediador.

La presencia de estos compuestos oxidados fue insignificante en los filtrados de la madera pretratada si enzima ni mediador (control), mientras que se observaron claramente en los filtrados después del tratamiento con lacasa sola y, especialmente en los filtrados obtenidos

después del pretratamiento con el sistema lacasa-MeS. Los principales compuestos oxidados de bajo peso molecular identificados incluyeron aldehídos simples (vainillina y siringaldehído) y ácidos (ácidos vainíllico y siríngico), y confirman que el tratamiento con el sistema lacasa-mediador consiste en una despolimerización oxidativa de la lignina, tal como se ha observado en el pretratamiento enzimático de los otros materiales lignocelulósicos estudiados en esa Tesis.

III.2. INTERACCIONES LIGNINA-CELULASAS

En este segundo apartado se describen los principales resultados del estudio de las interacciones entre la lignina y las celulasas usadas en la sacarificación, para investigar la inhibición que puede sufrir la enzima durante la hidrólisis enzimática de celulosa.

Para este estudio se aislaron las ligninas ("*milled wood lignin*", MWL) de diferentes materiales lignocelulóscios (pícea, paja de trigo y eucalipto), que presentan notables diferencias estructurales entre ellas. La lignina de pícea está compuesta casi exclusivamente por unidades de tipo G (99%), por el contrario, la lignina de eucalipto contiene mayoritariamente unidades de tipo S (70%), mientras que la lignina de la paja de trigo contiene aproximadamente el doble de unidades G que S (datos en el apartado III.1.1.). Estos datos fueron obtenidos mediante 2D-NMR **(Figura 24)** y ^{31}P NMR**.**

La interacción entra la lignina y las celulasas se estudió mediante Microgravimetría en Cristal de Cuarzo (*Quarz Cristal Microbalance*, QCM) y Resonancia de Plasmón Superficial (*Surface Plasmon Resonance*, SPR), después de formar un film cubierto con lignina. Estos instrumentos miden, mediante el peso y la resonancia respectivamente, las enzimas que se adhieren al film de lignina. Se utilizó un cóctel enzimático comercial formado por varias celulasas (Ctec2) y una celulasa purificada facilitada por VTT (Finlandia), la exoglucanasa de *Trichoderma reesei* (CBH-I). Se observó una fuerte adsorción de la enzima sobre los distintos films (**Figura 25**). Comparando ambas enzimas, se observó que la enzima purificada mostró menor afinidad a la adsorción sobre el film cubierto de lignina y

mayor reversibilidad de unión. Se observó una importante relación entre la unión enzimática y la relación S/G de la lignina. La afinidad más alta en la adsorción lignina-enzima se produjo en el caso de pícea, con un mayor contenido en unidades de tipo G (99% unidades G) mientras que la adsorción más baja se produjo en el caso de la lignina de eucalipto, con una mayor porcentaje de unidades de tipo S (70% unidades S).

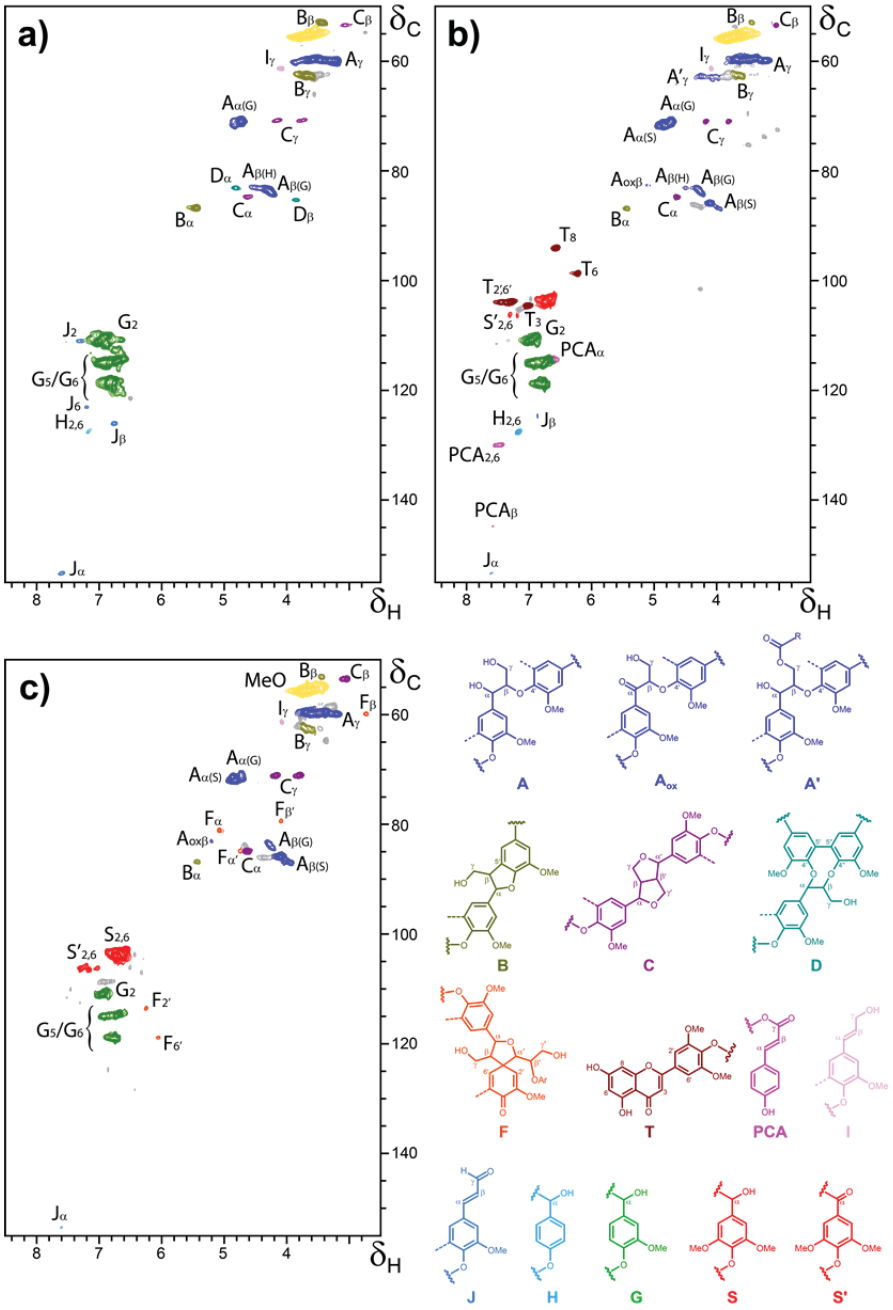


Figura 24. Regiones alifáticas y aromáticas de los espectros HSQC 2D-NMR de las diferentes ligninas utilizadas: a) pícea, b) paja de trigo y c) eucalipto. Estructura de los enlaces y unidades de lignina identificadas en los espectros HSQC.

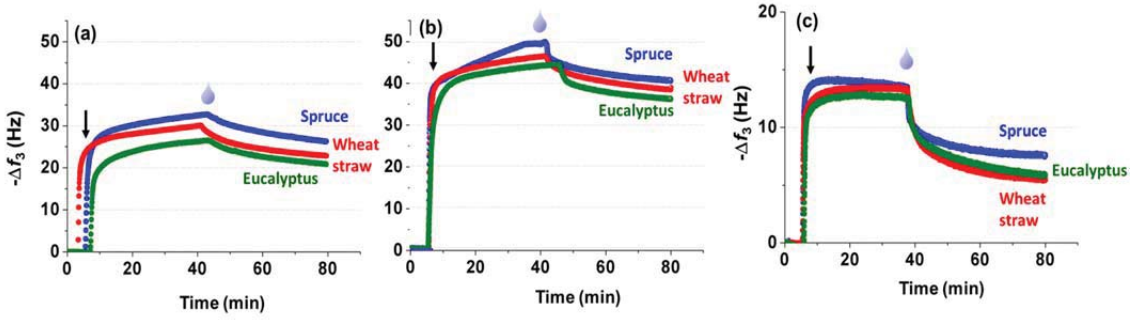


Figura 25. QCM tras la inyección de CTec2 en films de MWL. Se usaron dos concentraciones de enzimas diferentes, (a) 1 y (b) 5 mg/mL. (c) incluye el sensograma para CBH-I agregado a una concentración de 1 mg/mL. En cada figura, la flecha de la izquierda indica el tiempo aproximado en el que se inyectó la enzima después del equilibrio del film, y el símbolo de "caída" representa el momento en que la solución de enzima se reemplazó con solución de electrolito de fondo (etapa de enjuague).

Con el SPR se estudiaron los efectos de las interacciones electrostáticas en esta unión. Se compararon las ligninas de pícea y paja de trigo a distintas concentraciones del tampón buffer (50 y 200 mM), lo que hace que la lignina adquiera diferentes cargas (**Figura 26)**. A la concentración de 200 mM la lignina es menos negativa y el film de lignina absorbe más celulasas.

También se estudió la diferente reversibilidad de la adsorción de la enzima a diferentes concentraciones de tampón, observando que la carga iónica afecta significativamente en esta reversibilidad. En la lignina de pícea la fracción de enzima que fue adsorbida irreversiblemente fue del 38% a 50 mM y 50% a 200 mM, mientras que en el caso de la paja de trigo fue del 33% y 50%, respectivamente.

Este estudio demostró que lignina aislada de diferentes materias primas tiene un efecto negativo en la hidrólisis enzimática al producir una inhibición de las celulasas por la adsorción de estas.

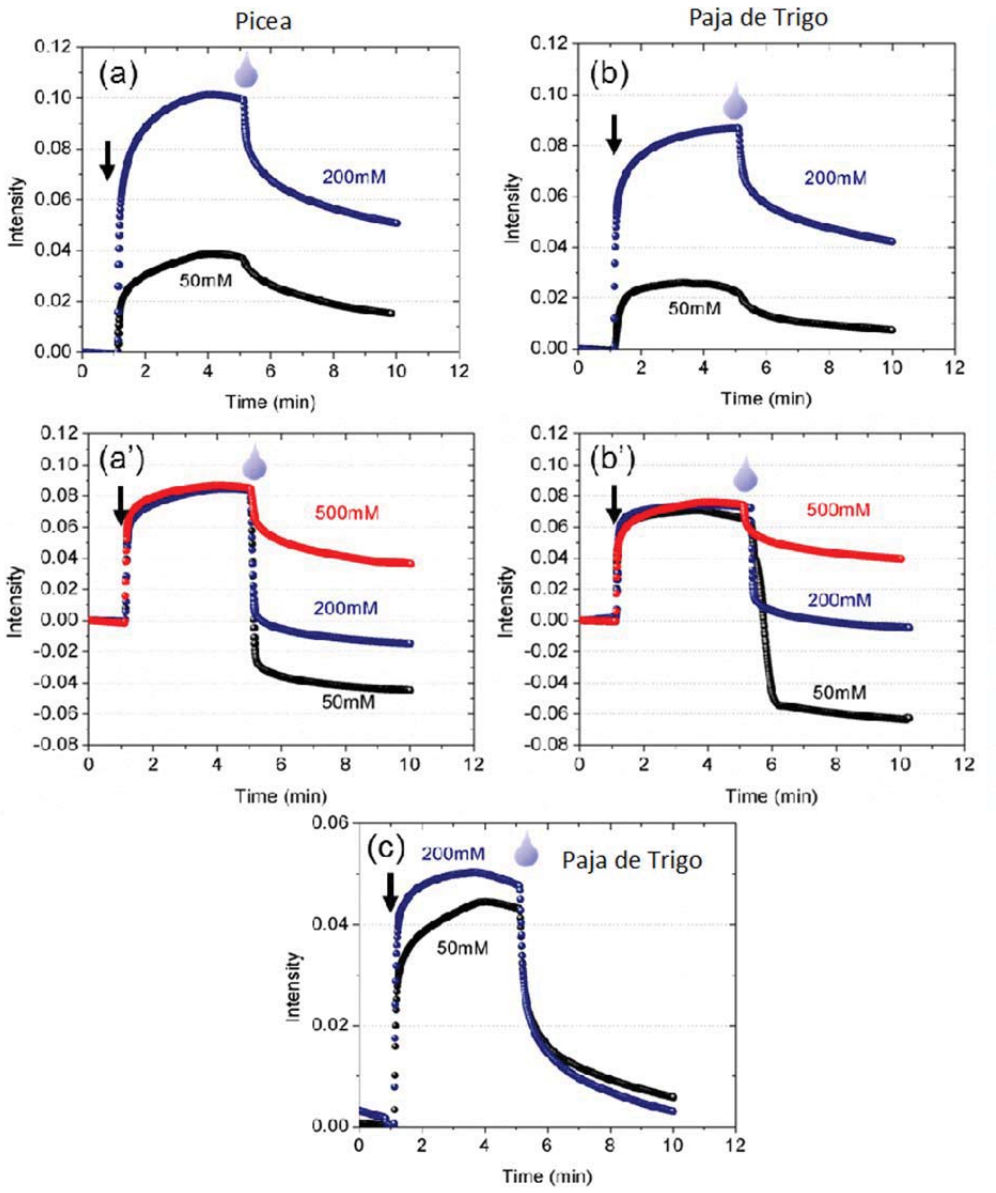


Figura 26. Sensograma de SPR que estudia la adsorción de Ctec2 (5mg/mL) disuelto en soluciones a diferentes concentraciones 50 mM y 200 mM, sobre ambas MWLs. La enzima se introdujo en el minuto 1 y en el minuto 5 se produce el lavado con solución tampón a las correspondientes concentraciones.

En conclusión, los diversos estudios realizados en la presente Tesis han demostrado el gran potencial del sistema lacasa-mediador como pretratamiento para la deslignificación de diferentes materiales lignocelulósicos con la consiguiente mejora en la posterior sacarificación enzimática con vistas a mejorar el rendimiento en bioetanol. Por otro lado, también se han demostrado los efectos negativos de la lignina en la inhibición de las celulasas.

IV. REFERENCIAS

Cultivos de eucalipto (*Eucalyptus globulus*)

Abdel-Hamid A.M., Solbiati J.O., Cann I.K. 2013. Insights into lignin degradation and its potential industrial applications. Advances in Applied Microbiology 82: 1-28.

Aden A., Ruth M., Ibsen K., Jechura J., Neeves K., Sheehan J., Wallace B. 2002. Lignocellulosic Biomass to Ethanol Process Design and Economics Utilizing Co-Current Dilute Acid Prehydrolysis and Enzymatic Hydrolysis for Corn Stover. Technical Report NREL/TP-510-32438. National Renewable Energy Laboratory. Golden, Colorado, US.

Adler E. 1977. Lignin chemistry-past, present and future. Wood Science and Technology. 11: 169-218.

Aitken Y., Cadel F., Voillot C. 1988. Constituants Fibreux des Pates Papiers et Cartons: Pratique de l'analyse. Centre Technique du Papier. St Martin d'Hères : Ecole Francaise de Papèterie et des Industries Graphiques. Paris, Francia.

Alizadeh H., Teymouri F., Gilbert T.I., Dale B.E. 2005. Pretreatment of switchgrass by ammonia fiber explosion (AFEX). Applied Biochemistry and Biotechnology 124: 1133-1141.

Alvira P., Tomás-Pejó E., Ballesteros M., Negro M.J. 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. Bioresource Technology 101: 4851-4861.

Arai T., Kosugi A., Chan H., Koukiekolo R., Yukawa H., Inui M., Doi R.H. 2006. Properties of cellulosomal family 9 cellulases from *Clostridium cellulovorans*. Applied Microbiology and Biotechnology 71: 654-660.

Avellar B.K., Glasser W.G. 1998. Steam-assisted biomass fractionation. I. Process considerations and economic evaluation. Biomass and Bioenergy 14: 205-218.

Babot E.D., Rico A., Rencoret J., Kalum L., Lund H., Romero J., del Río J.C., Martínez, A.T., Gutiérrez, A., 2011. Towards industrially feasible delignification and pitch removal by treating paper pulp with *Myceliophthora thermophila* laccase and a phenolic mediator. Bioresource Technology. 102, 6717-6722.

Balakshin M., Capanema, Gracz H., Chang H.M., Jameel H. 2011. Quantification of lignin-carbohydrate linkages with high-resolution NMR spectroscopy. Planta 233: 1097-1110.

Bali G., Meng X., Deneff J.I., Sun Q., Ragauskas A.J. 2015. The effect of alkaline pretreatment methods on cellulose structure and accessibility. ChemSusChem 8: 275-279.

Binod P., Sindhu R., Singhania R.R., Vikram S., Devi L., Nagalakshmi S., Kurien N., Sukumaran R.K., Pandey A. 2010. Bioethanol production from rice straw: an overview. Bioresource Technology 101: 4767-4774.

Bland D.E. 1966. Colorimetric and chemical identification of lignins in different parts of *Eucalyptus botryoides* and their relation to lignification. Holzforschung 20: 12-21.

Boerjan W., Ralph J., Baucher M. 2003. Lignin biosynthesis. Annual Review of Plant Biology 54: 519-546.

Bourbonnais R., Paice M.G. 1990. Oxidation of non-phenolic substrates: an expanded role for laccase in lignin biodegradation. FEBS Letters 267: 99-102.

Brunow G. 2005. Methods to reveal the structure of lignin. Biopolymers Online. Wiley-VCH Verlag GmbH & Co. KGaA. Weinheim, Alemania.

Call H., Mücke I. 1997. History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems (Lignozym®-process). Journal of Biotechnology 53: 163-202.

Carvalheiro F., Duarte L. C., Gírio F.M. 2008. Hemicellulose biorefineries: a review on biomass pretreatments. Journal of Scientific & Industrial Research 67: 849-864.

Chandra R., Bura R., Mabee W.E., Berlin A., Pan X., Saddler J.N. 2007. Substrate pretreatment: The key to effective enzymatic hydrolysis of lignocellulosics? Biofuels 108: 67-93.

Chen F., Tobimatsu Y., Jackson L., Nakashima J., Ralph J., Dixon R.A. 2013. Novel seed coat lignins in the Cactaceae: structure, distribution and implications for the evolution of lignin diversity. The Plant Journal 73: 201-211.

Christiernin M., Ohlsson A.B., Berglund T., Henriksson G. 2005. Lignin isolated from primary walls of hybrid aspen cell cultures indicates significant differences in lignin structure between primary and secondary cell wall. Plant Physiology and Biochemistry 43: 777-785.

Déjardin A., Laurans F., Arnaud D., Breton C., Pilate G., Leplé J-C. 2010. Wood formation in angiosperms. Biologies 333: 325-334.

del Río J.C., Gutiérrez A., Hernando M., Landín P., Romero J., Martínez A.T. 2005. Determining the influence of eucalypt lignin composition in paper pulp yield using Py-GC/MS. Journal of Analytical and Applied Pyrolysis 74: 110-115.

del Río J.C., Marques G., Rencoret J., Martínez A.T., Gutiérrez A. 2007. Occurrence of naturally acetylated lignin units. Journal of Agricultural and Food Chemistry 55: 5461-5468.

del Rio J.C., Rencoret J., Marques G., Gutiérrez A., Ibarra D., Santos J.I., Jiménez-Barbero J., Zhang L., Martínez A.T. 2008. Highly acylated (acetylated and/or *p*-coumaroylated) native lignins from diverse herbaceous plants. Journal of Agricultural and Food Chemistry 56: 9525-9534.

del Río J.C., Rencoret J., Prinsen P., Martínez A.T., Ralph J., Gutiérrez A. 2012. Structural characterization of wheat straw lignin as revealed by analytical pyrolysis, 2D-NMR, and reductive cleavage methods. Journal of Agricultural and Food Chemistry 60: 5922-5935.

del Río J.C., Lino A.G., Colodette J.L., Lima C.F., Gutiérrez A., Martínez A.T., Lu F., Ralph J., Rencoret J. 2015. Differences in the chemical structure of the lignins from sugarcane bagasse and straw. Biomass and Bioenergy 81: 322-338.

del Río J.C., Prinsen P., Cadena E.M., Martínez A.T., Gutiérrez A., Rencoret J. 2016. Lignin-Carbohydrate complexes from sisal (*Agave sisalana*) and abaca (*Musa textilis*): chemical composition and structural modifications during the isolation process. Planta. 243: 1143-1158.

del Río J.C., Rencoret J., Gutiérrez A., Kim H., Ralph J. 2017. Hydroxystilbenes are monomers in palm fruit endocarp lignins. Plant Physiology 174: 2072-2082.

Ding S.Y., Liu Y.S., Zeng Y., Himmel M.E., Baker J.O., Bayer E.A. 2012. How does plant cell wall nanoscale architecture correlate with enzymatic digestibility? Science 338: 1055-1060.

Ek M., Gellerstedt G., Henriksson G. 2009. Wood Chemistry and Biotechnology. Walter de Gruyter. Stockholm, Suecia.

Eriksson T., Börjesson J., Tjerneld F. 2002. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. Enzyme and Microbial Technology 31: 353-364.

Fengel D., Wegener G. 1983. Wood: Chemistry, Ultrastructure, Reactions. Walter de Gruyter. Berlin, Alemania.

Foyle T., Jennings L., Mulcahy P. 2007. Compositional analysis of lignocellulosic materials: Evaluation of methods used for sugar analysis of waste paper and straw. Bioresource Technology 98: 3026-3036.

Fukushima K., Terashima N. 1991. Heterogeneity in formation of lignin. Wood Science and Technology 25: 371-381.

García Hortal J.A. 2007. Fibras papeleras. Ediciones UPC (Universitat Politécnica de Catalunya), Barcelona , España.

Gutiérrez A., del Río J.C., Martínez M.J., Martínez A.T. 2001. The biotechnological control of pitch in paper pulp manufacturing. Trends in Biotechnology. 19: 340-348.

Gutiérrez A., del Río J.C., Ibarra D., Rencoret J., Romero J., Speranza M., Camarero S., Martínez M.J., Martínez A.T. 2006. Enzymatic removal of free and conjugated sterols forming pitch deposits in environmentally sound bleaching of eucalypt paper pulp. Environmental Science and Technology 40: 3416–3422.

Gutiérrez A., del Río J.C., Martínez A.T. 2009. Microbial and enzymatic control of pitch in the pulp and paper industry. Applied Microbiology and Biotechnology 82: 1005-1018.

Gutiérrez A., Rencoret J., Cadena E.M., Rico A., Barth D., del Río J.C., Martínez A.T. 2012. Demonstration of laccase-based removal of lignin from wood and non-wood plant feedstocks. Bioresource Technology 119: 114-122.

Hardell H., Leary G.J., Stoll M., Westermark U. 1980a. Variations in lignin structure in defined morphological parts of birch. Svensk Papperstidn 83: 71-74.

Hardell H., Leary G.J., Stoll M., Westermark U. 1980b. Variations in lignin structure in defined morphological parts of birch. Svensk Papperstidn 83: 44-49.

Harris D., DeBolt S. 2010. Synthesis, regulation and utilization of lignocellulosic biomass. Plant Biotechnology Journal 8: 244-262.

Hayes D.J. 2009. An examination of biorefining processes, catalysts and challenges. Catalysis Today 145: 138-151.

Higuchi T. 2012. Biochemistry and Molecular Biology of Wood. Springer Science & Business Media. London, UK.

Himmel M.E., Ding S.Y., Johnson D.K., Adney W.S., Nimlos M.R., Brady J.W., Foust T.D. 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 315: 804-807.

Jones J., Semrau K. 1984. Wood hydrolysis for ethanol production—previous experience and the economics of selected processes. Biomass 5: 109-135.

Kamm B., Gruber P.R., Kamm M. 2007. Biorefineries–Industrial Processes and Products. Weinheim: Wiley-Verlay Gmbtt and Co KGaA.

Karhunen P., Rummakko P., Sipila J., Brunow G., Kilpeläinen I. 1995. Dibenzodioxocins; a novel type of linkage in softwood lignins. Tetrahedron Letters 36: 169-170.

Karlen S.D., Smith R.A., Kim H., Padmakshan D., Bartuce A., Mobley J.K., Free H.C., Smith B.G., Harris P.J., Ralph J. 2017. Highly decorated lignins in leaf tissues of the Canary Island date palm *Phoenix canariensis*. Plant Physiology 175: 1058-1067.

Keshwani D.R. 2009. Microwave Pretreatment of Switchgrass for Bioethanol Production. Thesis Dissertation. North Carolina State University, US.

Kim H., Ralph J., Lu F., Ralph S.A., Boudet A.M., Mackay J.J., Sederoff R.R., Ito T., Kawai S., Ohashi H., Higuchi T. 2003. NMR analysis of lignins in CAD-deficient plants. Part 1. Incorporation of

hydroxycinnamaldehydes and hydroxybenzaldehydes into lignins. Organic & Biomolecular Chemistry 1: 268-281.

Kim S.M., Dien B.S., Tumbleson M.E., Rausch K.D., Singh V. 2016. Improvement of sugar yields from corn stover using sequential hot water pretreatment and disk milling. Bioresource Technology 216: 706-713.

Kirk, T.K., Farrell, R.L., 1987. Enzymatic "combustion": the microbial degradation of lignin. Annual Review of Microbiology 41, 465-505.

Kumar P., Barret D.M., Delwiche M.J., Stroeve P. 2009. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Industrial & Engineering Chemistry Research 48: 3713-3729.

Leplé J.-C., Dauwe R., Morreel K., Storme V., Lapierre C., Pollet B., Naumann A., Kang K.Y., Kim H., Ruel K., Lefèbvre A., Joseleau J.P., Grima-Pettenati J., de Rycke R., Andersson-Gunnerås S., Erban A., Fehrle I., Petit-Conil M., Kopka J., Polle A., Messens E., Sundberg B., Mansfield S.D., Ralph J., Pilate G., Boerjan W. 2007. Downregulation of cinnamoyl-coenzyme A reductase in poplar: multiple-level phenotyping reveals effects on cell wall polymer metabolism and structure. Plant Cell 19: 3669-3691.

Lourenço A., Rencoret J., Chemetova C., Gominho J., Gutiérrez A., del Río J.C., Pereira H. 2016. Lignin composition and structure differs between xylem, phloem and phellem in *Quercus suber L*. Frontiers in Plant Science 7: 1612.

Mansfield S.D., Mooney C., Saddler J. 1999. Substrate and enzyme characteristics that limit cellulose hydrolysis. Biotechnology Progress 15: 804-816.

Martínez Á.T., Rencoret J., Marques G., Gutiérrez A., Ibarra D., Jiménez-Barbero J., del Río J.C. 2008. Monolignol acylation and lignin structure in some nonwoody plants: a 2D NMR study. Phytochemistry 69: 2831-2843.

Moilanen U., Kellock M., Galkin S., Viikari L. 2011. The laccase-catalyzed modification of lignin for enzymatic hydrolysis. Enzyme and Microbial Technology 49: 492-498.

Moilanen U., Kellock M., Várnai A., Andberg M., Viikari L. 2014. Mechanisms of laccase-mediator treatments improving the enzymatic hydrolysis of pre-treated spruce. Biotechnology for Biofuels 7: 177.

Nakagame S., Chandra R.P., Saddler J.N. 2010. The effect of isolated lignins, obtained from a range of pretreated lignocellulosic substrates, on enzymatic hydrolysis. Biotechnology and Bioengineering 105: 871-879.

Nakagame S., Chandra R.P., Kadla J.F., Saddler J.N. 2011. The isolation, characterization and effect of lignin isolated from steam pretreated Douglas-fir on the enzymatic hydrolysis of cellulose. Bioresource Technology 102: 4507-4517.

Oliva J.M., Sáez F., Ballesteros I., González A., Negro M.J., Manzanares P., Ballesteros M. 2003. Effect of lignocellulosic degradation compounds from steam explosion pretreatment on ethanol fermentation by thermotolerant yeast *Kluyveromyces marxianus*. Applied Microbiology and Biotechnology 105: 141-154.

Palmqvist E., Almeida J.S., Hahn-Hägerdal B. 1999. Influence of furfural on anaerobic glycolytic kinetics of *Saccharomyces cerevisiae* in batch culture. Biotechnology and Bioengineering 62: 447-454.

Palmqvist E., Hahn-Hägerdal B. 2000. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresource Technology 74: 25-33.

Palonen H., Tjerneld F., Zacchi G., Tenkanen M. 2004a. Adsorption of *Trichoderma reesei* CBH I and EG II and their catalytic domains on steam pretreated softwood and isolated lignin. Journal of Biotechnology 107: 65-72.

Palonen, H., Viikari, L., 2004b. Role of oxidative enzymatic treatments on enzymatic hydrolysis of softwood. Biotechnology and Bioengineering 86, 550-557.

Parthasarathi R., Romero R.A., Redondo A., Gnanakaram S. 2011. Theoretical study of the remarkably diverse linkages in lignin. The Journal of Physical Chemistry Letters 2: 2660-2666.

Poppius-Levlin K., Wang W., Tamminen T., Hortling B., Vikari L., Niku-Paavola M.L. 1999. Effects of laccase/HBT treatment on pulp and lignin structures. Journal of Pulp and Paper Science 25: 90-94.

Prasad N.K., Vindal V., Narayana S.L., Kunal S.P., Srinivas M. 2012. In silico analysis of *Pycnoporus cinnabarinus* laccase active site with toxic industries dyes. Journal of Molecular Modeling. 18: 2013-2019.

Rabemanolontsoa H. y Saka S. 2012. Holocellulose determination in biomass. Zero-Carbon Energy Kyoto 2011. Green Energy and Technology. Springer: 135-140.

Ragauskas A.J., Beckham G.T., Biddy M.J., Chandra R., Chen F., Davis M.F., Davison B.H., Dixon R.A., Gilna P., Keller M., Langan P., Naskar A.K., Saddler J.N., Tschaplinski T.J., Tuskan G.A., Wyman C.E. 2014. Lignin valorization: improving lignin processing in the biorefinery. Science 344: 1246843-1246852 .

Rahikainen J.L., Martín-Sampedro R., Heikkinen H., Rovio S., Marjamaa K., Tamminen T., Rojas O.J., Kruus K. 2013. Inhibitory effect of lignin during cellulose bioconversion: the effect of lignin chemistry on non-productive enzyme adsorption. Bioresource Technology 133: 270-278.

Ralph J., Lundquist K., Brunow G., Lu F., Kim H., Schatz P.F., Marita J.M., Hatfield R.D., Ralph S.A., Christensen J.H., Boerjan W. 2004. Lignins: natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids. Phytochemistry Reviews. 3: 29-60.

Rencoret J., Marques G., Gutiérrez A., Nieto L., Jiménez-Barbero J., Martínez A.T., del Río J.C. 2009. Isolation and structural characterization of the milled-wood lignin from *Paulownia fortunei* wood. Industrial Crops and Products. 30: 137-143.

Rencoret J., Gutiérrez A., Nieto L., Jiménez-Barbero J., Faulds C.B., Kim H., Ralph J., Martínez Á.T., del Río J.C. 2010. Lignin composition and structure in young versus adult *Eucalyptus globulus* plants. Plant Physiology 155: 667-682.

Rencoret J., Ralph J., Marques G., Gutiérrez A., Martínez Á.T., del Río J.C. 2013. Structural characterization of lignin isolated from coconut

(*Cocos nucifera*) coir fibers. Journal of Agricultural and Food Chemistry 61: 2434-2445.

Rencoret J., Pereira A., del Río J.C., Martínez Á.T., Gutiérrez A. 2016. Laccase-mediator pretreatment of wheat straw degrades lignin and improves saccharification. BioEnergy Research 9: 917-930.

Rencoret J., Pereira A., del Río J.C., Martínez Á.T., Gutiérrez A. 2017. Delignification and saccharification enhancement of sugarcane byproducts by a laccase-based pretreatment. ACS Sustainable Chemistry & Engineering 5: 7145-7154.

Rencoret J., Kim H., Evaristo A.B., Gutiérrez A., Ralph J., del Río J.C. 2018. Variability in lignin composition and structure in cell walls of different parts of macaúba (*Acrocomia aculeata*) palm fruit. Journal of Agricultural and Food Chemistry 66; 138-153.

Rico A., Rencoret J., del Río J.C., Martínez Á.T., Gutiérrez A. 2014a. Pretreatment with laccase and a phenolic mediator degrades lignin and enhances saccharification of Eucalyptus feedstock. Biotechnology for Biofuels 7: 6.

Rico, A., Rencoret, J., del Río, J.C., Martínez, A.T., Gutiérrez, A., 2014b. In-depth 2D NMR study of lignin modification during pretreatment of eucalyptus wood feedstock with laccase and mediators. BioEnergy Research 8: 211-230.

Riva S. 2006. Laccases: blue enzymes for green chemistry. Trends in Biotechnology 24: 219-226.

Ruiz-Dueñas, F.J., Martínez, A.T., 2009. Microbial degradation of lignin: How a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this. Microbial Biotechnology 2: 164-177.

Saini J.K., Saini R., Tewari L. 2015. Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: concepts and recent developments. 3 Biotech 5: 337-353.

Sarkanen K.V., Ludwig C.H. 1971. Lignins. Occurrence, Formation, Structure, and Reactions. Wiley-Interscience. New York, US.

Scheller H.V., Ulvskov P. 2010. Hemicelluloses. Annual Review of Plant Biology 61: 263-289.

Sjöström E., Westermark U. 1999. Chemical composition of wood and pulps: basic constituents and their distribution. Analytical Methods in Wood Chemistry, Pulping, and Papermaking. Springer**:** 1-19. Berlin, Alemania.

Sjöstrom E. 2013. Wood chemistry: Fundamentals and Applications. Academic Press. San Diego, US.

Studer, M.H., DeMartini, J.D., Davis, M.F., Sykes, R.W., Davison, B., Keller, M., Tuskan, G.A., Wyman, C.E., 2011. Lignin content in natural Populus variants affects sugar release. Proceedings of the National Academy of Science USA 108: 6300-6305.

Sun R. 2010. Cereal Straw as a Resource for Sustainable Biomaterials and Biofuels: Chemistry, Extractives, Lignins, Hemicelluloses and Cellulose. Elsevier. Amsterdam, Holanda.

Sun Y., Cheng J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technology 83: 1-11.

Taherzadeh M.J., Karimi K. 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. International Journal of Molecular Sciences 9: 1621-1651.

Ten E., Vermerris W. 2015. Recent developments in polymers derived from industrial lignin. Journal of Applied Polymer Science 132: 42069.

Teymouri F., Laureano-Pérez L., Alizadeh H., Dale B.E. 2005. Optimization of the ammonia fiber explosion (AFEX) treatment parameters for enzymatic hydrolysis of corn stover. Bioresource Technology 96: 2014-2018.

Trzcinski A.P., Stuckey D.C. 2015. Contribution of acetic acid to the hydrolysis of lignocellulosic biomass under abiotic conditions. Bioresource Technology 185: 441-444.

Vanhala P., Bergström I., Haaspuro T., Kortelainen P., Holmberg M., Forsius M. 2016. Boreal forests can have a remarkable role in reducing greenhouse gas emissions locally: land use-related and anthropogenic greenhouse gas emissions and sinks at the municipal level. Science of The Total Environment 557: 51-57.

Velmurugan R., Muthukumar K. 2011. Utilization of sugarcane bagasse for bioethanol production: sono-assisted acid hydrolysis approach. Bioresource Technology 102: 7119-7123.

Vohra M., Manwar J., Manmode R., Patil S.V. 2014. Bioethanol production: feedstock and current technologies. Journal of Environmental Chemical Engineering 2: 573-584.

Wan C., Li Y. 2012. Fungal pretreatment of lignocellulosic biomass. Biotechnology Advances 30: 1447-1457.

Widsten, P., Kandelbauer, A., 2008. Laccase applications in the forest products industry: a review. Enzyme and Microbial Technology 42: 293-307.

Wyman C.E., Dale B.E., Elander R.T., Holtzapple M., Ladisch M.R., Lee Y.Y. 2005. Coordinated development of leading biomass pretreatment technologies. Bioresource Technology 96: 1959-1966.

Yachmenev V., Condon B., Klasson T., Lambert A. 2009. Acceleration of the enzymatic hydrolysis of corn stover and sugar cane bagasse celluloses by low intensity uniform ultrasound. Journal of Biobased Materials and Bioenergy 3: 25-31.

Young C.-Y., Ni S.P., Fan K.S. 2010. Working towards a zero waste environment in Taiwan. Waste Management & Research 28: 236-244.

Zabed H., Sahu J.N., Suely A., Boyce A.N., Faruq G. 2017. Bioethanol production from renewable sources: current perspectives and technological progress. Renewable and Sustainable Energy Reviews. 71: 475-501.

Zeng Y., Zhao S., Yang S., Ding S-Y. 2014. Lignin plays a negative role in the biochemical process for producing lignocellulosic biofuels. Current Opinion in Biotechnology 27: 38-45.

Zhang L., Gellerstedt G. 2001. NMR observation of a new lignin structure, a spiro-dienone. Chemical Communications 24: 2744-2745.

Zhao X., Chen K., Liu D. 2009. Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis. Applied Microbiology and Biotechnology 82: 815-827.

V. PUBLICACIONES

Cultivos de *Picea abies*

PUBLICACIÓN 1:

Rencoret J., Pereira A., del Río J.C., Martínez A.T., Gutiérrez A. (2016) Laccase-mediator pretreatment of wheat straw degrades lignin and improves saccharification. *BioEnergy Research* 9: 917-93.

Bioenerg. Res. (2016) 9:917–930
DOI 10.1007/s12155-016-9745-z

Laccase-Mediator Pretreatment of Wheat Straw Degrades Lignin and Improves Saccharification

Jorge Rencoret[1] · Antonio Pereira[1] · José C. del Río[1] · Angel T. Martínez[2] · Ana Gutiérrez[1]

Published online: 12 May 2016

Abstract Agricultural by-products such as wheat straw are attractive feedstocks for the production of second-generation bioethanol due to their high abundance. However, the presence of lignin in these lignocellulosic materials hinders the enzymatic hydrolysis of cellulose. The purposes of this work are to study the ability of a laccase-mediator system to remove lignin improving saccharification, as a pretreatment of wheat straw, and to analyze the chemical modifications produced in the remaining lignin moiety. Up to 48 % lignin removal from ground wheat straw was attained by pretreatment with *Pycnoporus cinnabarinus* laccase and 1-hydroxybenzotriazole (HBT) as mediator, followed by alkaline peroxide extraction. The lignin removal directly correlated with increases (~60 %) in glucose yields after enzymatic saccharification. The pretreatment using laccase alone (without mediator) removed up to 18 % of lignin from wheat straw. Substantial lignin removal (37 %) was also produced when the enzyme-mediator pretreatment was not combined with the alkaline peroxide extraction. Two-dimensional nuclear magnetic resonance (2D NMR) analysis of the whole pretreated wheat straw material swollen in dimethylsulfoxide-d_6 revealed modifications of the lignin polymer, including the lower number of aliphatic side chains involved in main β-*O*-4′ and β-5′ inter-unit linkages per aromatic lignin unit. Simultaneously, the removal of *p*-hydroxyphenyl, guaiacyl, and syringyl lignin units and of *p*-coumaric and ferulic acids, as well as a moderate decrease of tricin units, was observed without a substantial change in the wood polysaccharide signals. Especially noteworthy was the formation of Cα-oxidized lignin units during the enzymatic treatment.

Keywords Enzymatic delignification · Laccase-mediator · Lignin · Wheat straw · 2D NMR · Bioethanol

Introduction

Agricultural and forestry residues represent an enormous source of readily available biomass for biofuel production without the need for additional land cultivation. Among agricultural residues, wheat straw is potentially one of the most favorable feedstocks in terms of the quantity of biomass available [1]. However, in spite of the availability of these residues or other lignocellulosic biomass, their variable composition and recalcitrance represent some technical and economic challenges. Cellulose, hemicelluloses, and lignin are the three main components of lignocellulosic biomass linked into a complex matrix highly resistant to chemical and biological conversion. These components are more resistant to being broken down and fermented than starch and sucrose in the conventional food crops, making the conversion processes more complicated. Biofuel production from lignocellulosic material requires deconstruction of the cell wall into individual polymers and hydrolysis of the carbohydrates into monomeric sugars. One of the major factors causing biomass recalcitrance toward saccharification is correlated with the content and composition of lignin [2–4].

Lignin is a three-dimensional polymer constituted by phenylpropanoid subunits linked together by different ether and carbon-carbon bonds. Lignin is intimately interlaced with

Jorge Rencoret and Antonio Pereira contributed equally to this work.

✉ Ana Gutiérrez
anagu@irnase.csic.es

[1] Instituto de Recursos Naturales y Agrobiología de Sevilla, CSIC, Reina Mercedes 10, 41012 Seville, Spain

[2] Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, 28040 Madrid, Spain

hemicelluloses in the plant cell wall forming a matrix to cover the largely crystalline cellulose microfibrils. Its aromatic nature and complex structure make lignin degradation very difficult. Lignin has been shown to have a detrimental effect on the hydrolysis of biomass because it physically hinders the access of cellulases and also binds them reducing activity [5]. Therefore, biomass pretreatment to remove lignin is essential for the enzymatic hydrolysis of lignocellulose. Biotechnology can contribute to plant biomass deconstruction by providing biocatalysts being able to degrade or modify lignin [6]. Due to the complex structure of lignin, including heterogeneity of the monomers and linkages, which are not hydrolyzable, these biocatalysts must be oxidative (i.e., oxidoreductases) and also non-specific. Among these, laccases seem to be the most suitable enzymes for industrial application, because they only require dioxygen as oxidant and also because it can be produced on a large scale [7].

Laccases (phenoloxidases, EC 1.10.3.2) are multicopper oxidases that oxidize substituted phenols using molecular oxygen as the final electron acceptor. The direct action of laccases on lignin is, in principle, restricted to the phenolic units that only represent a small percentage of the total polymer [8], a fact that limits their biotechnological application. However, the discovery that some synthetic compounds can act as electron carriers between the enzyme and the final substrate [9], 1-hydroxybenzotriazole (HBT) being among the most efficient ones [10], has expanded the utility of laccases. A number of studies have confirmed the potential of laccase-mediator systems for paper pulp delignification [11, 12], pitch control [13], polymer modification [14], and other applications in the forest industry [15] and bioethanol production from pretreated lignocellulose [16–18]. Recently, the ability of fungal laccases to remove lignin (when applied in combination with redox mediators) from whole [19, 20] and ensiled [21] lignocellulosic biomass, making cellulose accessible to hydrolysis, was reported. Additionally, the use of laccases in bioethanol production has also been reported as a biotechnological tool for the removal of phenolic inhibitors generated during steam explosion of lignocellulosic feedstocks [22], although some recent work also shows that some laccase-derived compounds can affect negatively the enzymatic hydrolysis [23].

A previous work on the laccase-mediator treatment of acid-pretreated wheat straw has been reported [17]. The novelty of the pretreatment described here, based on the use of a fungal laccase from the basidiomycete *Pycnoporus cinnabarinus* [24] in combination with HBT as mediator [25], is that it was applied directly in the ground wheat straw feedstock (without a previous chemical pretreatment). Additionally, in the present study, the modification of lignin structure in the pretreated lignocellulosic material was analyzed in depth by two-dimensional nuclear magnetic resonance (2D NMR) spectroscopy of the whole sample at the gel state [26, 27]. Besides lignin modification and removal, the effect of the laccase-mediator on the saccharification yield from the pretreated wheat straw feedstock was also assessed.

Material and Methods

Lignocellulosic Feedstock, Enzyme, and Mediator

Wheat straw (*Triticum durum* var. Carioca) was harvested from an experimental field in Seville (Spain). Wheat straw samples were air-dried and grounded in an IKA MF10 cutting mill to pass through a 100-mesh screen and then finely milled using a Retsch PM100 planetary mill at 400 rev min^{-1} (with 5-min breaks after every 5 min of milling) using a 500-mL agate jar and agate ball bearings (20 × 20 mm). The total ball-milling time for the samples was 5 h. The chemical composition of wheat straw feedstock (as % dry weight) was as follows: glucose, 39.4 ± 0.7; xylose, 16.0 ± 0.3; arabinose, 3.8 ± 0.2; soluble acid lignin, 1.5 ± 0.1; and Klason lignin, 16.0 ± 0.3.

The laccase was provided by INRA (Marseille, France) and was obtained from a laccase-hyperproducing strain of the fungus *P. cinnabarinus* (Herpoël et al. 2000). Its activity was measured as initial velocity during oxidation of 5 mM ABTS from Roche to its cation radical (ε_{436} 29300 M^{-1} cm^{-1}) in 0.1 M sodium acetate (pH 5) at 24 °C. The laccase activity of the enzyme preparation was 102 U/ml (specific activity 156 U/mg). One activity unit (U) was defined as the amount of enzyme transforming 1 μmol of ABTS per minute. HBT from Sigma-Aldrich (Steinheim, Germany) was used as mediator.

Laccase-Mediator Treatments

The wheat straw samples were treated with the *P. cinnabarinus* laccase in the presence (and absence) of HBT, as mediator. Laccase doses of 13 and 65 U g^{-1} were assayed, together with 5, 10, and 20 % HBT, all referred to straw dry weight. The treatments were carried out in 200-mL pressurized bioreactors (Labomat, Mathis) placed in a thermostatic shaker at 170 rev min^{-1} and 50 °C, using 4-g (dry weight) samples at 6 % (*w*/*w*) solid loading in 50 mM sodium tartrate buffer (pH 4) under O_2 atmosphere (2 bar) for 24 h. Additionally, the treatment with laccase (65 U g^{-1}) and HBT (20 %) was also performed in the presence of 0.05 % Tween 20, to test the effect of adding a surfactant in both the enzymatic delignification and enzymatic hydrolysis of wheat straw [28]. After the treatment, the samples were filtered through a Büchner funnel and washed with 1 L of water. In some cases, a subsequent alkaline peroxide extraction step was performed after the enzymatic pretreatment. In this case, enzymatically treated samples at 6 % (*w*/*w*) solid loading were submitted to a peroxide-reinforced

alkaline extraction using 1 % (*w*/*w*) NaOH and 3 % (*w*/*w*) H_2O_2 (also with respect to sample dry weight) at 80 °C for 90 min, followed by water washing [29]. The solid loading for the latter step was achieved by determining the moisture content of an aliquot. Treatments with laccase (65 U g^{-1}) alone (without mediator) and controls without laccase and mediator were also performed (followed in both cases by the corresponding alkaline peroxide extraction). A control with mediator alone was not included taking into account the results from previous studies. Duplicate experiments of a representative (65 U g^{-1} laccase and 20 % HBT) laccase-mediator treatment (including control, laccase alone and laccase-HBT) were performed to estimate the variability in biological replicates (as shown in Table 1 footnote). A one-way analysis of variance (ANOVA) was conducted to compare the effects of the different enzymatic treatments on the lignin removal and on the releases of glucose and xylose. Post hoc pairwise comparisons, using the Tukey HSD test, were performed in order to determine which means are significantly different from each other. Klason lignin content was estimated (in triplicate measurements) according to T222 om-88 [30]. The data from both biological and technical replicates were averaged. Weight loss (%) was determined for all the treatments (Table 1) with respect to the control without enzyme-mediator and alkaline extraction: laccase (65 U g^{-1}), 2.3 %; laccase (65 U g^{-1})-HBT (20 %), 6.1 %; control/alkaline peroxide, 0.8 %; laccase (65 U g^{-1})/alkaline extraction, 4.8 %; laccase (13 U g^{-1})-HBT (10 %)/alkaline extraction, 7.3 %; laccase (65 U g^{-1})-HBT (5 %)/alkaline extraction, 7.7 %; laccase (65 U g^{-1})-HBT (20 %)/alkaline extraction,13.4 %; and laccase (65 U g^{-1})-HBT (20 %)/T20/alkaline extraction, 13.5 %. The weight loss of control without enzyme-mediator and alkaline extraction with respect to initial wheat straw was 12.7 %.

Saccharification of Treated Wood

The laccase-pretreated samples were hydrolyzed with a cocktail containing commercial enzymes (from Novozymes, Bagsvaerd) with cellulase (Celluclast 1.5 L; 2 filter paper units (FPU) g^{-1}) and β-glucosidase (Novozyme 188; 6 U g^{-1}) activities, at 1 % solid loading in 3 mL of 100 mM sodium citrate (pH 5) for 72 h at 45 °C, in a thermostatic shaker at 170 rev min^{-1} (in triplicate experiments). The specific activities of Celluclast 1.5 L and β-glucosidase are 700 EGU/g and 250 CBU/g, respectively.

The different monosaccharides released were determined as alditol acetates [31] by GC. An HP 5890 gas chromatograph (Hewlett-Packard, Hoofddorp, The Netherlands) equipped with a split-splitless injector and a flame ionization detector was used. The injector and detector temperatures were set at 225 and 250 °C, respectively. Samples were injected in the split mode (split ratio 10:1). Helium was used as the carrier gas. The capillary column used was a DB-225 (30 m × 0.25 mm i.d.,

Table 1 Lignin content and monosaccharide release (% sample weight) by cellulase hydrolysis of wheat straw samples

Wheat straw samples	Lignin	Glucose	Xylose
Initial wheat straw	16.0 ± 0.3	21.6 ± 0.2	6.9 ± 0.1
- Laccase-mediator:			
Control	15.6 a ± 0.2	24.1 a ± 0.0	7.3 a ± 0.4
Laccase (65 U g^{-1})	15.0 a ± 0.1	29.0 b ± 0.1	9.4 b ± 0.1
Laccase (65 U·g^{-1})-HBT (20 %)	9.8 b ± 0.3	33.6 c ± 0.2	11.2 c ± 0.2
- Laccase-mediator/alkaline peroxide:			
Control	14.8 a ± 0.2	28.4 a ± 0.4	9.8 a ± 0.3
Laccase (65 U g^{-1})	12.1 b ± 0.3	31.1 b ± 0.3	9.8 a ± 0.2
Laccase (13 U g^{-1})-HBT (10 %)	9.4 c ± 0.1	35.1 c ± 0.5	11.4 b ± 0.6
Laccase (65 U g^{-1})-HBT (5 %)	9.2 c ± 0.4	36.5 d ± 0.3	11.5 b ± 0.6
Laccase (65 U g^{-1})-HBT (20 %)	7.7 d ± 0.2	41.5 e ± 0.7	13.3 c ± 0.2
Laccase (65 U g^{-1})-HBT (20 %)/T20	7.5 d ± 0.2	45.5 f ± 0.6	13.7 c ± 0.0

Lignin content (as Klason lignin, corrected for ash) and monosaccharides from cellulase hydrolysis of wheat straw samples treated with the following: (i) *P. cinnabarinus* laccase (13 and 65 U g^{-1}) and HBT mediator (10 and 20 %); (ii) the same previous enzymatic treatment (i) followed by an alkaline peroxide extraction including a treatment in presence of Tween 20 (T20) and (ii) compared with a control without enzyme, a treatment with laccase alone, and the initial wheat straw sample. Biological duplicates for a representative laccase-mediator treatment (including 65 U g^{-1} laccase and 20 % HBT) were separately analyzed showing that the 95 % confidence intervals of the biological duplicates are smaller than the differences found between the control, laccase alone, and laccase-HBT treatments (data not shown). In the table, means ± SD from technical triplicates of experiments representative for the different treatments are provided. Letters next to the means, from Tukey test, show whether or not the results from different treatments (shown in each column) are significantly different from each other, at the 0.05 level (means with the same letters are not significantly different, whereas means followed by different letters are significantly different)

0.15-μm film thickness; Agilent J&W). The oven was temperature-programmed from 220 °C (held for 5 min) to 230 °C (held for 5 min) at 2 °C min^{-1}. Peaks were quantified by area, and glucose and xylose were used as standards to elaborate calibration curves. The data from both biological and technical replicates were averaged.

2D NMR Spectroscopy

For gel-state NMR experiments, ~70 mg of finely divided (ball-milled) wheat straw samples was directly transferred into 5-mm NMR tubes and swelled in 1 mL of deuterated dimethylsulfoxide (DMSO-d_6), forming a gel inside the NMR tube [26, 27].

Heteronuclear single-quantum correlation (HSQC) 2D-NMR spectra were acquired at 25 °C on a Bruker AVANCE III 500-MHz spectrometer fitted with a cryogenically cooled 5-mm TCI gradient probe with inverse geometry (proton coils closest to the sample). The 2D ^{13}C-^{1}H correlation spectra were carried out using an adiabatic HSQC pulse program (Bruker standard pulse sequence "hsqcetgpsisp2.2"), which enabled a semiquantitative analysis of the different ^{13}C-^{1}H correlation signals. Spectra were acquired from 10 to 0 ppm (5000 Hz) in F2 (^{1}H) using 1000 data points for an acquisition time of 100 ms, an interscan delay of 1 s, and from 200 to 0 ppm (25,168) in F1 (^{13}C) using 256 increments of 32 scans, for a total acquisition time of 2 h 34 min. The $^{1}J_{CH}$ used was 145 Hz. Processing used typical matched Gaussian apodization in ^{1}H and a squared cosine bell in ^{13}C. The central solvent peak was used as an internal reference (δ_C/δ_H 39.5/2.49). The ^{13}C-^{1}H correlation signals from the aromatic region of the spectrum were used to estimate the content of lignin, *p*-coumaric acid, ferulic acid, and tricin (compared with the amorphous polysaccharide content, estimated from the anomeric xylose and glucose signals) and the lignin composition in terms of G, S, and oxidized S (S') units. Correlations in the aliphatic-oxygenated region were used to estimate the inter-unit linkage and end-unit abundances in lignin. The intensity corrections introduced by the adiabatic pulse program permit to refer the side-chain integrals to the previously obtained number of lignin units.

Results and Discussion

Delignification of Wheat Straw by Laccase-HBT

Wheat straw lignin is a guaiacyl-rich lignin [32] that is usually reported to be more resistant to degradation than the syringyl type [33]. For this reason, and also based on results from previous studies, a high-redox potential laccase (from the basidiomycete *P. cinnabarinus*) and mediator (HBT) were selected for wheat straw delignification.

Enzymatic pretreatments using different doses of *P. cinnabarinus* laccase (13 and 65 U g^{-1}) and HBT (5, 10, and 20 %) were carried out. The selection of these doses was based on previous studies on the pretreatment of another non-woody feedstock (elephant grass) [19] with a lignin having also a high proportion of guaiacyl units although not as high as that of wheat straw [32, 34]. Additionally, the effect of the combination of the enzymatic treatment with a subsequent alkaline peroxide extraction was studied. The lignin content (as Klason lignin) of wheat straw samples after the pretreatments was determined (Table 1). The effect of oxygen (used in the enzymatic reactions) and alkaline peroxide extraction in wheat straw delignification can be observed by comparing the corresponding controls with the initial straw.

The lignin content of wheat straw samples after the laccase-mediator pretreatment using *P. cinnabarinus* laccase (65 U g^{-1}) in the presence of HBT (20 %) decreased about 37 % with respect to the corresponding control (treated under the same conditions except the addition of enzyme and mediator) (Table 1). The pretreatment with laccase alone (in the absence of HBT) only decreased the lignin content by 5 %. Similar values of lignin content decrease have been reported after laccase (alone) treatment of several lignocellulosic feedstocks, as steam-pretreated giant reed and Northern spruce [35].

The laccase alone is not very efficient for delignification because of its low oxidation potential and for stearic hindrance reasons. The relatively low oxidation potential of laccases only allows them to oxidize phenolic groups in lignin.

Combination of the enzymatic pretreatment with a subsequent alkaline peroxide extraction step (removing partially degraded lignin) increased wheat straw delignification up to 48 and 18 % (with respect to lignin content of the control sample) in the pretreatments with laccase-HBT and laccase alone, respectively (Table 1). With the aim of exploring if the enzymatic delignification could be enhanced by the presence of a surfactant, as reported for pulp biobleaching [36], addition of Tween 20 was tested, but only a modest effect on the delignification of wheat straw was observed. When the enzymatic pretreatment (followed by alkaline peroxide extraction) was carried out under the same reaction conditions and dose of laccase, but with lower dose of HBT (5 %), 38 % reduction of lignin content (with respect to the control sample) was attained, and a similar value (37 % delignification) was obtained when both the laccase and mediator doses were reduced to 13 U g^{-1} enzyme and 10 % HBT, respectively.

Similar delignification degrees to those above described (with lower laccase-mediator doses) were obtained after four sequential laccase-HBT treatments (including four alkaline peroxide extraction steps) of elephant grass [19]. Interestingly, when the latter pretreatment was applied to eucalypt wood, higher delignification degrees (up to 48 %) were attained [19, 37, 38], showing that some woody feedstocks can be competitive for bioethanol production. The better delignification values obtained in the latter studies can be

related with the predominance of syringyl lignin units in eucalypt wood with respect to elephant grass and wheat straw [32, 34, 39, 40]. On the other hand, no decrease in lignin content was obtained by other authors after treating steam-exploded wheat straw with *P. cinnabarinus* laccase and HBT [41].

Enzymatic Hydrolysis of Pretreated Wheat Straw

The wheat straw samples treated with laccase alone and in the presence of HBT and the corresponding controls (and the initial untreated wheat straw) were hydrolyzed (72 h) using a cellulase and β-glucosidase cocktail [19], and the main monosaccharides released (glucose and xylose) were analyzed by GC.

At low cellulase (2 FPU g^{-1}) and β-glucosidase (6 U g^{-1}) doses, increases in glucose and xylose yields up to 40 and 47 % (with respect to controls), respectively, were attained in the samples pretreated with 65 U g^{-1} of laccase and 20 % mediator (Table 1). Interestingly, in the samples pretreated with laccase alone, 24–25 % increases in glucose and xylose release were produced, although the Klason lignin content was not significantly lowered. The positive effect of laccase (alone) in enzymatic saccharification has been attributed to the binding of laccase to lignin in lignocellulosic substrates that competes with and consequently reduces the non-specific binding of cellulases to lignin improving saccharification [17], in addition to some delignification effect on the phenolic lignin units. Moreover, a surface analysis of lignocellulose substrate after treatment with *Trametes* laccase revealed an increase in carboxylic acid residues after laccase treatment and suggested that this enzymatic modification to lignin may decrease the non-specific adsorption of negatively charged cellulases [16].

On the other hand, increases up to 46 and 9 % in glucose and 35 and 0 % in xylose yields, respectively, were produced when the enzymatic pretreatments of laccase-HBT and laccase alone were combined with an alkaline peroxide extraction step (the highest increases being attained with the highest doses of laccase, 65 U g^{-1}, and HBT, 20 %). Moreover, a similar improvement in saccharification yield was produced after the laccase-mediator treatment alone (without a subsequent alkaline extraction) unlike in a recent study of a laccase-HBT treatment of acid-pretreated wheat straw [17]. In the pretreatment with 13 U g^{-1} of laccase, in combination with 10 % mediator (followed by alkaline extraction), the increases in glucose and xylose yields were 24 and 16 % (with respect to control), respectively (Table 1). Similarly, an increase in glucose and xylose yields up to 29 and 17 %, respectively, was produced using 65 U g^{-1} of laccase, in combination with only 5 % mediator (followed by alkaline extraction). The above glucose release values are higher than those recently reported by other authors in the treatment with laccase-HBT of acid-pretreated wheat straw [17] in which higher laccase doses were used but also higher solid loading in enzymatic hydrolysis. Other authors have reported no improvement in saccharification yields after different laccase-mediator treatments of steam-exploded wheat straw [41]. On the other hand, the saccharification yields shown here after laccase-mediator pretreatment of wheat straw are higher than those reported for other pretreatments of wheat straw like steam explosion [42]. The sugar degradation and generation of inhibitory compounds during steam explosion that affect the hydrolysis can explain the lower saccharification yields attained in steam-exploded wheat straw. Additionally, it should be mentioned the higher solid loading used by these authors that also may affect enzymatic saccharification.

Finally, noteworthy was the improvement in glucose and xylose yields (up to 60 and 40 %, respectively) obtained in the present study when a surfactant (Tween 20) was added during the laccase-HBT pretreatment (Table 1). Since the addition of Tween 20 did not affect the delignification of wheat straw (as shown above) and, in contrast, enhanced the saccharification, its effect may be related with an increase of cellulase action on cellulose [28].

Enzymatic Modification of Wheat Straw Lignin (as Shown by 2D NMR)

The modification of lignin structure produced by the enzymatic pretreatments of wheat straw was studied by 2D NMR. With this purpose, the whole wheat straw samples were swelled in DMSO-d_6 forming a gel and analyzed by HSQC NMR. The main lignin structures identified are shown in Fig. 1, and the different lignin signals assigned on the spectra are listed in Table 2. The HSQC spectra of the different straw samples are provided in Figs. 2, 3, and 4. The composition of lignin, in terms of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, and the *p*-coumaric acid and ferulic acid contents, together with the relative abundance of the main inter-unit linkages in the different samples, estimated from the NMR signal volume integrals, are shown in Tables 3 and 4.

Effect of the Laccase-Mediator Treatment

Figure 2 shows the HSQC spectra of the wheat straw samples after pretreatment with laccase (65 U g^{-1}) in the presence (Fig. 2c, f) and absence (Fig. 2b, e) of HBT (20 %) and the corresponding control (Fig. 2a, d). The aliphatic-oxygenated region of the HSQC spectrum of the control without enzyme (Fig. 2a) shows signals of lignin and carbohydrates, the latter mainly corresponding to amorphous xylan (X), acetylated xylan (X'), and glucan (Gl) units, since crystalline cellulose is nearly "silent" in lignocellulose gel spectra under solution NMR conditions. In this region, signals of methoxyls and side chains in β-*O*-4′ alkyl-aryl ether lignin substructures (A),

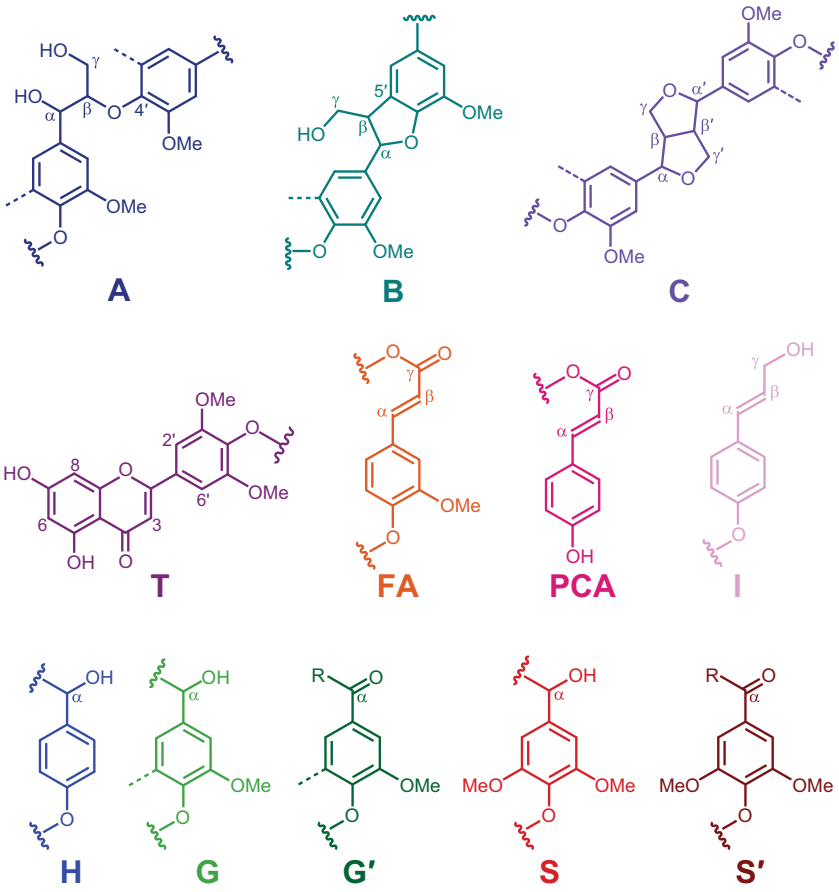


Fig. 1 Main lignin structures identified in the wheat straw samples analyzed by HSQC NMR (Figs. 2, 3, and 4). *A* β-*O*-4′ ether (including a second S or G unit), *B* phenylcoumaran, *C* resinol, *T* tricin, *PCA* *p*-coumaric acid, *FA* ferulic acid (esterified with hemicellulose sugars), *I* cinnamyl alcohol end group, *H* *p*-hydroxyphenyl unit, *G* guaiacyl unit, *S* syringyl unit, *G′* C_α-oxidized G unit, and *S′* C_α-oxidized S unit (R in G′ and S′ can be a hydroxyl in carboxylic acids or a lignin side chain in ketones)

including C_α/H_α, C_β/H_β, and C_γ/H_γ correlations (A_α, A_β, and A_γ, respectively), were observed. The C_α/H_α and C_β/H_β correlations gave two different signals corresponding to β-*O*-4′ substructures where the second unit is an S unit or a G unit ($A_{\alpha(S)}$ and $A_{\alpha(G)}$, and $A_{\beta(S)}$ and $A_{\beta(G)}$, respectively). The A_γ signal overlaps with related signals in lignin and other lignocellulose constituents. Other less prominent signals corresponding to phenylcoumaran (B) and resinol (C) substructures were also observed in the HSQC spectrum. The main signals in the aromatic/unsaturated region of the HSQC spectrum (Fig. 2d) correspond to the benzenic rings and unsaturated side chains of H, G, and S lignin units; *p*-coumaric acid (PCA); and ferulic acid (FA). Additionally, several signals corresponding to the flavone tricin (T) recently reported in wheat-straw lignin [32] were observed. The S-lignin units showed a prominent signal for the $C_{2,6}/H_{2,6}$ correlation ($S_{2,6}$), while the G-lignin units showed different correlations for C_2/H_2 (G_2), C_5/H_5 (G_5), and C_6/H_6 (G_6). From the integrals of the above signals, an S/G ratio around 0.4 and a large predominance of β-*O*-4′ ether linkages, together with some phenylcoumarans and resinols, were estimated for lignin in wheat straw feedstock, in agreement with previous studies [32]. A low-intensity signal corresponding to $C_{2,6}/H_{2,6}$ correlation in H units ($H_{2,6}$) was also observed. On the other hand, the PCA prominent signals in this region corresponded to the $C_{2,6}/H_{2,6}$ ($PCA_{2,6}$) and $C_{3,5}/H_{3,5}$ ($PCA_{3,5}$) aromatic correlations and the C_α/H_α (PCA_α) and C_β/H_β (PCA_β) olefinic correlations. Additionally, two signals corresponding to C_2/H_2 and C_6/H_6 correlations in FA (FA_2 and FA_6, respectively) were also observed, while the other aromatic and olefinic signals of the FA overlapped with similar signals of PCA and lignin G units.

The HSQC spectra of the wheat straw samples after the treatments with the higher laccase-mediator doses showed important differences compared to the control ones. While the carbohydrate signals remained unchanged, most of the lignin (and cinnamic acid) signals were strongly decreased with respect to the initial and control samples (Table 3). The prominent signals of side chains in β-*O*-4′ lignin substructures (A), present in the control spectrum (Fig. 2a), as well as B and C signals were not observed after the enzymatic treatment (Fig. 2c). On the other hand, the signals of lignin units (H, G, and S) present in the spectrum of the control sample (Fig. 2d) also disappeared (Fig. 2f), except the very low-intensity S signal. Moreover, the signals of cinnamates (PCA and FA) also disappeared, whereas, interestingly, the flavonoid structure, tricin, seemed to be more resistant to laccase-mediator treatments (Fig. 2f).

Table 2 Assignments of lignin ^{13}C-^{1}H correlation signals in the 2D HSQC spectra of wheat straw samples

Label	δ_C/δ_H (ppm)	Assignment
B_β	53.1/3.41	C_β/H_β in phenylcoumaran substructures (**B**)
C_β	53.5/3.06	C_β/H_β in β-β′ resinol substructures (**C**)
$-OCH_3$	55.5/3.72	C/H in methoxyls
A_γ	59.6/3.37 and 3.71	C_γ/H_γ in γ-hydroxylated β-*O*-4′ substructures (**A**)
I_γ	61.5/4.09	C_γ/H_γ in cinnamyl alcohol end groups (**I**)
X_5	62.9/3.16 and 3.87	C_5/H_5 in xylopyranose units
$A_{\alpha(G)}$	71.1/4.71	C_α/H_α in β-*O*-4′ substructures (**A**) linked to a G-unit
C_γ	71.2/4.17	C_γ/H_γ in β-β′ resinol substructures (**C**)
$A_{\alpha(S)}$	71.5/4.81	C_α/H_α in β-*O*-4′ substructures (**A**) linked to a S-unit
X_2	72.5/3.03	C_2/H_2 in xylopyranose units
X'_2	73.1/4.49	C_2/H_2 in 2-*O*-acetylated xylopyranose units
X_3	73.8/3.23	C_3/H_3 in xylopyranose units
X'_3	74.7/4.79	C_3/H_3 in 3-*O*-acetylated xylopyranose units
X_4	75.2/3.50	C_4/H_4 in xylopyranose units
$A_{\beta(G)}$	83.5/4.35	C_β/H_β in β-*O*-4′ substructures linked (**A**) to a G unit
C_α	84.7/4.63	C_α/H_α in β-β′ resinol substructures (**C**)
$A_{\beta(S)}$	85.9/4.05	C_β/H_β in β-*O*-4′ substructures linked (**A**) to a S unit
B_α	86.8/5.42	C_α/H_α in phenylcoumaran substructures (**B**)
T_8	94.0/6.56	C_8/H_8 in tricin units (**T**)
T_6	98.8/6.20	C_6/H_6 in tricin units (**T**)
X'_1	99.3/4.48	C_1/H_1 in 3-*O*-acetylated xylopyranose units
X_1/X'_1	101.5/4.26	C_1/H_1 in xylopyranose units
Gl_1	102.9/4.16	C_1/H_1 in glucopyranose units
$S_{2,6}$	103.8/6.69	C_2/H_2 and C_6/H_6 in etherified syringyl units (**S**)
$T_{2',6'}$	103.9/7.28	$C_{2'}/H_{2'}$ and $C_{6'}/H_{6'}$ in tricin units (**T**)
T_3	104.6/7.02	C_3/H_3 in tricin units (**T**)
$S'_{2,6}$	106.1/7.29, 106.2/7.18	C_2/H_2 and C_6/H_6 in α-oxidized syringyl units (**S'**)
G_2	110.7/6.96	C_2/H_2 in guaiacyl units (**G**)
G'_2	110.8/7.38	C_2/H_2 in α-oxidized guaiacyl units (**G'**)
FA_2	110.9/7.33	C_2/H_2 in ferulate (**FA**)
PCA_β/FA_β	113.5/6.29	C_β/H_β in *p*-coumarate (**PCA**) and ferulate (**FA**)
$H_{3,5}$	113.6/6.64	C_3/H_3 and C_5/H_5 in *p*-hydroxyphenyl units (**H**)
FA_5	114.1/6.77	C_5/H_5 in ferulate (**FA**)
G_5/G_6	114.9/6.78, 6.94 and 118.8/6.77	C_5/H_5 and C_6/H_6 in guaiacyl units (**G**)
G'_5	115.0/6.73	C_5/H_5 in α-oxidized guaiacyl units (**G'**)
$PCA_{3,5}$	115.3/6.76	C_3/H_3 and C_5/H_5 in *p*-coumarate (**PCA**)
FA_6	123.2/7.10	C_6/H_6 in ferulate (**FA**)
$H_{2,6}$	127.6/7.14	C_2/H_2 and C_6/H_6 in *p*-hydroxyphenyl units (**H**)
$PCA_{2,6}$	130.0/7.48	C_2/H_2 and C_6/H_6 in *p*-coumarate (**PCA**)
PCA_α/FA_α	145.0/7.58	C_α/H_α in *p*-coumarate (**PCA**) and ferulate (**FA**)

From Figs. 2, 3, and 4 HSQC spectra. See Fig. 1 for chemical structures

A prominent signal corresponding to $C_{2,6}/H_{2,6}$ correlations in C_α-oxidized S-lignin units ($S'_{2,6}$) appeared as a consequence of this treatment. Likewise, new signals, tentatively assigned to oxidized G-lignin units (G'), appeared in the spectrum. Generation of oxidized lignin structures is congruent with the nature of the lignin biodegradation process, which has been described as an "enzymatic combustion" where fungal oxidoreductases play a central role [43]. It is generally accepted that lignin degradation by white rot fungi and their ligninolytic peroxidases starts by aromatic ring oxidation to a cation radical but quickly leads to side-chain C_α-C_β cleavage causing depolymerization [44]. The same mechanism has been suggested for some laccase reactions mediated by synthetic compounds, e.g., 2,2-azinobis(3-ethylbenzothiazoline-

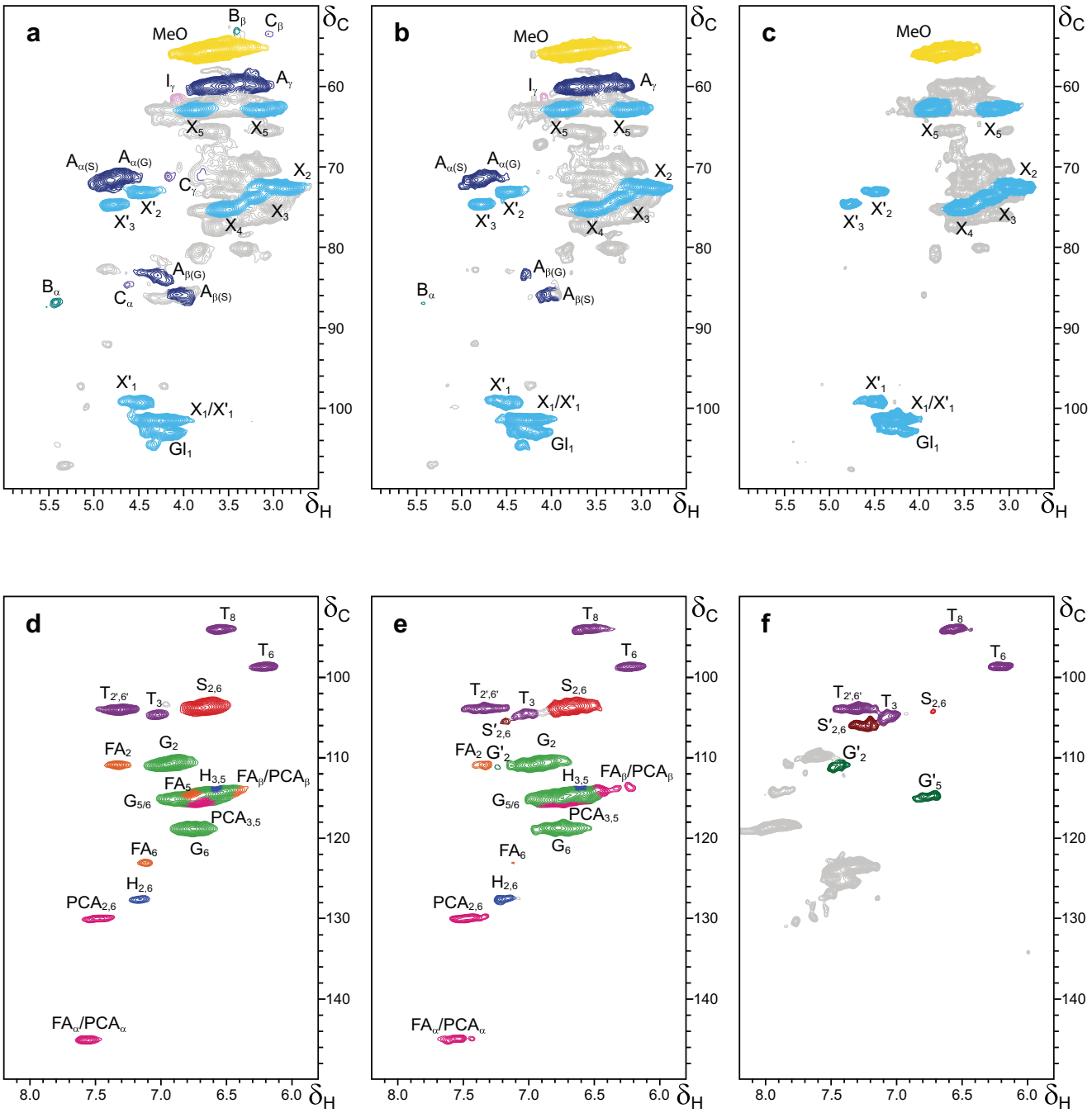


Fig. 2 HSQC NMR spectra of wheat straw after laccase-mediator treatment (without a subsequent alkaline peroxide extraction). Expanded aliphatic oxygenated (δ_H-δ_C, 2.5–6.0 and 50–110 ppm; *top*) and aromatic (δ_H-δ_C, 5.8–8.2 and 90–150 ppm; *bottom*) regions of the HSQC NMR spectra of wheat straw treated with *P. cinnabarinus* laccase-HBT: **a**, **d** control without enzyme; **b**, **e** 65 U g^{-1} enzyme; and **c**, **f** 65 U g^{-1} enzyme and 20 % HBT (see Table 2 for lignin signal assignment, Fig. 1 for the main lignin structures identified, and Table 3 for quantification of these lignin structures). Carbohydrate signals are also observed mainly corresponding to C_1–C_5 in normal (X_1–X_5) and acetylated xylan units (X'_1–X'_5) (an anomeric glucose signal was also identified, G_1) (unassigned signals in *gray*, including signals from the enzyme and the mediator)

6-sulfonate), but the action of laccase-HBT on non-phenolic lignin models is predominantly produced by hydrogen-atom abstraction from the C_α position, followed by alkyl-aryl ether breakdown [45, 46]. This attack mechanism would result in the increased amount of C_α-oxidized lignin units as observed after the laccase-mediator treatment of the wheat straw. Concerning C_α-oxidized groups, a higher proportion of S and G acid monomers with respect to the aldehyde counterpart has been determined by thermochemolysis in lignins extracted from laccase-mediator-treated wheat straw [17].

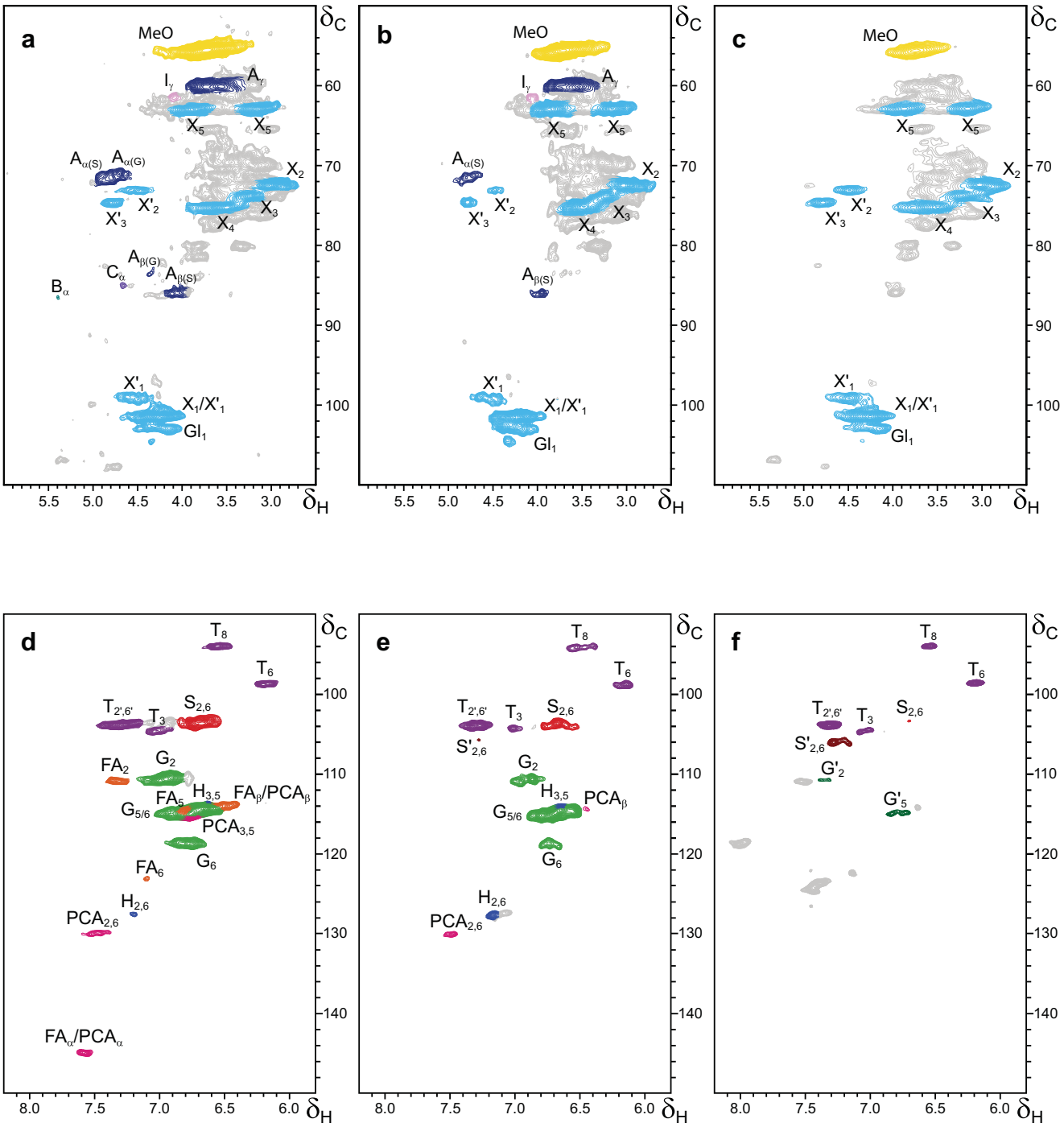


Fig. 3 HSQC NMR spectra of wheat straw after laccase-mediator treatment (higher doses) and alkaline peroxide extraction. Expanded aliphatic oxygenated (*top*) and aromatic (*bottom*) regions of the HSQC NMR spectra of wheat straw samples after treatment with laccase-HBT followed by an alkaline peroxide extraction: **a**, **d** Control without enzyme; **b**, **e** 65 U g^{-1} enzyme; and **c**, **f** 65 U g^{-1} enzyme and 20 % HBT (see Fig. 2 legend for additional information)

Remarkably, some lignin modification and removal were also shown by the NMR spectra of the wheat straw treated with laccase alone (Fig. 2b, e), with a relative decrease of the lignin signals compared to the carbohydrate signals and the initial and control samples (Table 3). Among them, the C signals disappeared, and A_α and A_β and B signals decreased considerably with respect to the control sample, although the changes were always less intense than those found in the sample treated with laccase and mediator. Concerning the aromatic units, the G lignin signals decreased slightly and C_α-oxidation of S (and G) units was observed to some extent although much less pronounced than in the presence of HBT. The lignin

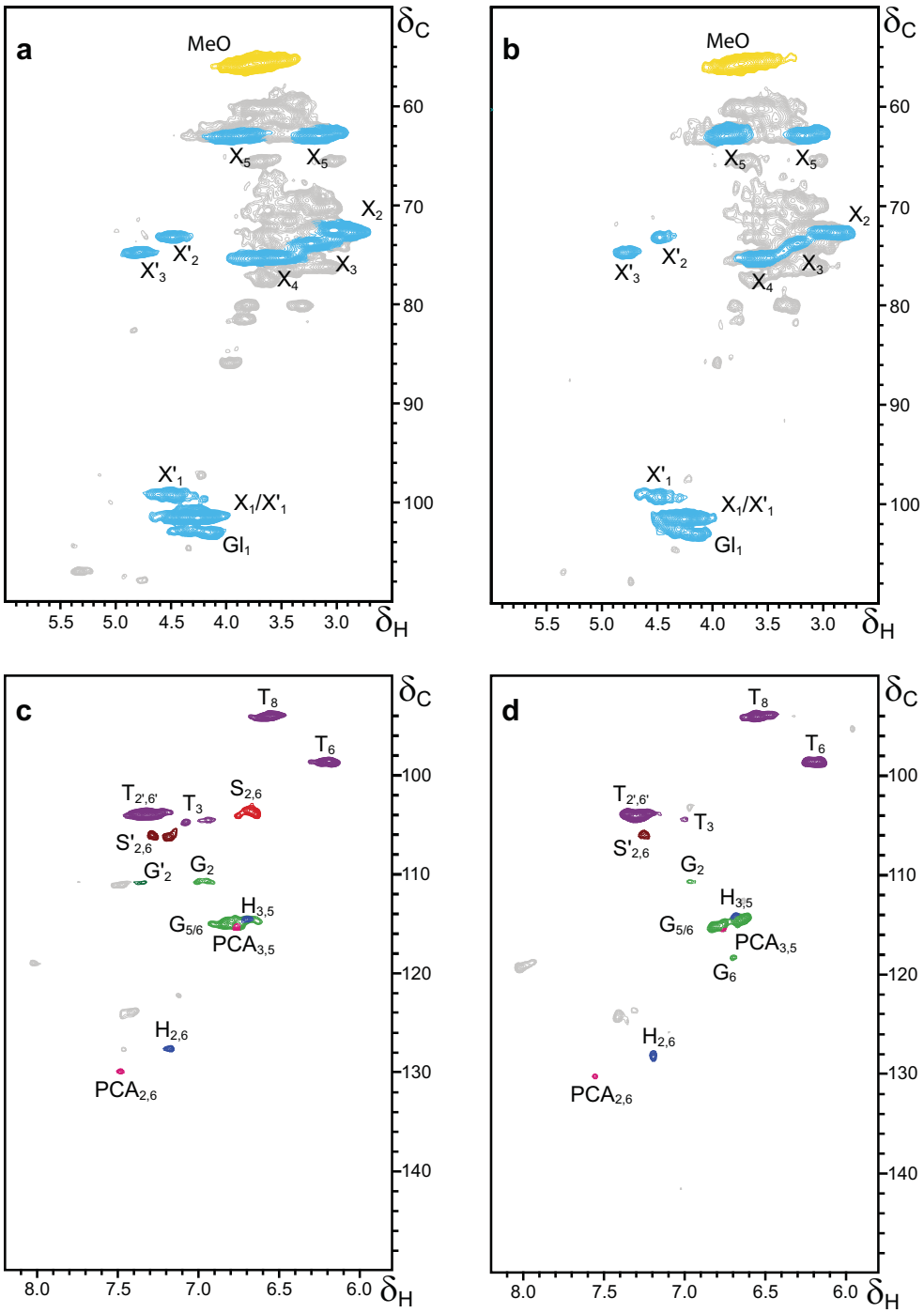


Fig. 4 HSQC NMR spectra of wheat straw after laccase-mediator treatment (lower doses) and alkaline peroxide extraction. Expanded aliphatic oxygenated (*top*) and aromatic (*bottom*) regions of the HSQC NMR spectra of wheat straw samples after treatment with laccase-HBT followed by an alkaline peroxide extraction: **a**, **b** 13 U g^{-1} enzyme and 10 % HBT and **c**, **d** 65 U g^{-1} enzyme and 5 % HBT (see Fig. 2 legend for additional information)

modification observed in the pretreatments with laccase alone could be due to the action of laccase catalyzing the oxidation of the phenolic moiety (less than 20 %) of wheat straw lignin since laccase alone is not able to catalyze the oxidation of non-phenolic lignin units. Reactions of high- and low-redox potential laccases with lignin models (monomers) have revealed that any laccase reactions are initiated through oxidation of the phenolic hydroxyl group and that laccase cannot directly oxidize the α-carbon [47]. This side-chain oxidation to an α-carbonyl leads (in reaction with monomers) to further coupling to the 4- or 5-position (in guaiacyl-type units). However, if the 5-position is occupied (as in syringyl-type groups), the side-chain oxidation to an α-carbonyl serves as a dead-end reaction with higher oxidation potential or to the coupling to the 1-position with subsequent breakage of the bond producing cleavage of the lignin polymer [47]. On the other hand, the cleavage of the C_{α}-C_{β} bond catalyzed by laccase has been shown in phenolic lignin model dimers by,

Table 3 NMR analysis of straw treated with laccase-HBT and controls (parentheses refer to total lignin)

	Initial	Control	Laccase	Laccase-HBT
- Sample composition[a]:				
Syringyl lignin units (**S**)	5.8 (30)	7.7 (31)	5.5 (30)	0.5 (7)
C_α-oxidized S units (**S'**)	0 (0)	0 (0)	0.4 (2)	2.2 (32)
Guaiacyl lignin units (**G**)	12.6 (63)	15.2 (63)	11.1 (60)	0.4 (6)
C_α-oxidized G units (**G'**)	0 (0)	0 (0)	0.5 (3)	3.4 (51)
p-Hydroxyphenyl units (**H**)	1.5 (7)	1.5 (6)	0.9 (5)	0.3 (4)
Total lignin	*19.9* (*100*)	*24.4* (*100*)	*18.4* (*100*)	*6.7* (*100*)
Tricin (**T**)	3.6 (18)	4.7 (19)	3.4 (19)	3.5 (52)
p-Coumaric acid (**PCA**)	1.3 (6)	1.8 (7)	1.5 (8)	0 (0)
Ferulic acid (**FA**)	3.4 (17)	3.8 (15)	2.0 (11)	0 (0)
Total aromatics	*28.1*	*34.6*	*25.3*	*10.2*
Sugar units	71.9	65.4	74.7	89.8
Total	100	100	100	100
Lignin S/G ratio	0.46	0.50	0.51	0.70
- Side chains and end groups[b]:				
β-*O*-4′ ethers (**A**)	80 (61)	82 (62)	88 (59)	0
Phenylcoumarans (**B**)	8 (6)	9 (7)	4 (3)	0
Resinols (**C**)	9 (7)	7 (5)	4 (3)	0
Cinnamyl end groups (**I**)	3 (3)	3 (2)	4 (3)	0
Total	100 (77)	100 (76)	100 (68)	0

Milled wheat straw was treated with laccase (65 U g^{-1}) in combination with HBT (20 %) and laccase alone and compared with control treatment (without enzyme) and initial material (spectrum not shown) using HSQC NMR (Fig. 2)

[a] Sample composition provides the molar amount of normal (S, G, and H) and C_α-oxidized (S' and G') lignin units, S/G ratio, tricin (T), *p*-coumaric acid (PCA), and ferulic acid (FA) from integration of aromatic signals and sugar units from integration of anomeric carbon signals, in the HSQC spectra (parentheses, values referred to total lignin); total lignin (italics)= S+S'+G+G'+H, total aromatics (italics)= total lignin+T+PCA+FA

[b] The percentages of lignin side chains forming different substructure (A-C) and cinnamyl alcohol end groups are provided from integration of aliphatic signals in the HSQC spectra (parentheses, values referred to total lignin)

first, oxidizing C_α and by splitting the aryl-alkyl bond [48]. These studies help to explain the lignin degradation observed in the pretreatments with laccase alone. However, it should be taken into account that these reactions occur much easier using model compounds than in polymeric lignin for steric reasons, among others. On the other hand, this lignin modification observed by the action of laccase alone could be also due to the action of natural occurring mediators that can be present in wheat straw.

Noteworthy in the treatment with laccase alone is the preferential decrease of FA signals, with respect to the PCA signals, that would be related to the lower redox potential of FA (due to the presence of an electron-donating substituent on the aromatic ring).

Effect of the Alkaline Peroxide Extraction and Laccase-Mediator Doses

The NMR spectra of the wheat straw treatments followed by an alkaline peroxide extraction are shown in Fig. 3, including control (Fig. 3a, d), laccase alone (Fig. 3b, e), and laccase-HBT (Fig. 3c, f) treatments, and the results from signal integration are provided in Table 4. The spectra of the latter samples (Fig. 3c, f) were similar to those of samples pretreated under the same conditions but without alkaline peroxide extraction (Fig. 2c, f), although with (slightly) lower-intensity aromatic signals (including C_α-oxidized lignin units) in agreement with lower lignin content (Table 1). The S'/S and G'/G ratios decreased after the alkaline peroxide extraction from 4.4 and 8.5 to 3.3 and 5.5, respectively. During alkaline peroxide extraction, the hydroperoxide anions produced are reported to react with the carbonyl structures existing in lignin resulting in C–C bond cleavage [17], and similar reaction has been reported in alkaline pulping [49].

Differences were also observed between the spectra of laccase-pretreated samples without and with an alkaline peroxide extraction. In the latter spectra, a high decrease in β-*O*-4′ side-chain substructures (Fig. 3b) was observed together with the complete disappearance of FA signals and the remarkable decrease of lignin units (Fig. 3e). Finally, some

Table 4 NMR analysis after laccase-HBT (different doses) treatment followed by alkaline extraction (parentheses refer to total lignin)

	Control	Laccase	Lac13-HBT10	Lac65-HBT5	Lac65-HBT20
- Sample composition[a]:					
Syringyl lignin units (**S**)	4.8 (28)	3.3 (30)	1.3 (24)	0.2 (8)	0.3 (10)
C_α-oxidized S units (**S'**)	0	0.3 (3)	1.0 (19)	0.6 (29)	1.0 (38)
Guaiacyl lignin units (**G**)	11.6 (68)	6.5 (58)	1.5 (28)	0.7 (37)	0.2 (6)
C_α-oxidized G units (**G'**)	0	0.3 (3)	1.1 (20)	0	1.1 (39)
p-Hydroxyphenyl units (**H**)	0.8 (5)	0.8 (7)	0.4 (8)	0.5 (26)	0.2 (7)
Total lignin	*17.2 (100)*	*11.1 (100)*	*5.3 (100)*	*1.9 (100)*	*2.7 (100)*
Tricin (**T**)	3.1 (18)	2.7 (25)	2.0 (38)	3.0 (154)	1.4 (50)
p-Coumaric acid (PCA)	1.4 (8)	0.8 (7)	0.3 (6)	0.3 (13)	0
Ferulic acid (FA)	2.6 (15)	0.3 (3)	0	0	0
Total aromatics	*24.2*	*15.0*	*7.7*	*5.1*	*4.1*
Sugar units	75.8	85.1	92.3	94.9	96.0
Total	100	100	100	100	100
Lignin S/G ratio	0.41	0.53	0.89	1.02	1.05
- Side chains and end groups[b]:					
β-*O*-4′ ethers (**A**)	76 (59)	91 (59)	100 (16)	29 (12)	0
Phenylcoumarans (**B**)	6 (5)	0	0	0	0
Resinols (**C**)	10 (8)	0	0	0	0
Cinnamyl end groups (**I**)	8 (6)	9 (6)	0	71 (30)	0
Total	100 (78)	100 (65)	100 (16)	100 (42)	0

Milled wheat straw was treated with 13 or 65 U g^{-1} of laccase (Lac13 and Lac65) in combination with 5, 10, or 20 % HBT (HBT5, HBT10, and HBT20, respectively) and compared with laccase alone and with a control without enzyme (followed in all cases by an extraction with alkaline peroxide) using HSQC NMR (Figs. 3 and 4)

[a] Sample composition provides the molar amount of normal (S, G, and H) and C_α-oxidized (S' and G') lignin units, S/G ratio, tricin (T), *p*-coumaric acid (PCA), and ferulic acid (FA) from integration of aromatic signals and sugar units from integration of anomeric carbon signals, in the HSQC spectra (parentheses, values referred to total lignin); total lignin (italics)= S+S'+G+G'+H, total aromatics (italics)= total lignin+T+PCA+FA

[b] The percentages of lignin side chains forming different substructure (A-C) and cinnamyl alcohol end groups are provided from integration of aliphatic signals in the HSQC spectra (parentheses, values referred to total lignin)

effect was also produced by the alkaline peroxide extraction itself. If we compare the NMR spectra of controls with (Fig. 3a, d) and without (Fig. 2a, d) alkaline peroxide extraction, it is evident that B and C signals decreased with the alkaline peroxide extraction. Likewise, the intensities of signals of side chains in β-*O*-4′ lignin, especially those where the second unit is a G unit, also decreased. In the aromatic region of the spectrum (Fig. 3d), the most noticeable effect of alkaline peroxide extraction was the decrease in FA. Interestingly, the hemicellulose was not deacetylated (unchanged X'_1, X'_2, and X'_3 signals) in agreement to the mild conditions of the alkaline extraction used.

Finally, interesting results were obtained when lower laccase and HBT doses were assayed, always followed by an alkaline peroxide extraction, including the following: (i) reduced mediator dose (to only 25 % of the initial) (Fig. 4b, d) and (ii) reduced both laccase (to 20 % of the initial) and mediator dose (to 50 % of the initial) (Fig. 4a, c). These two treatments seem to be very efficient in degrading/removing lignin (including β-*O*-4′ side chains and lignin units) similarly to the treatment with higher mediator doses, although the latter showed higher efficacy toward H lignin units, PCA, and tricin. In the three treatments, a preferential degradation of G units with respect to S ones was observed (Table 4). The increase in S/G ratio with the treatment was also revealed in previous studies dealing with laccase-mediator pretreatments of other lignocellulosic feedstocks [19, 37, 38]. On the other hand, in spite of similar lignin removal with both lower doses (Table 1), a higher decrease in S and G lignin units is shown by 2D NMR for the former one that is also correlated with a higher saccharification yield.

Conclusions

Wheat straw can be delignified by a basidiomycete laccase in the presence of HBT, directly on the ground lignocellulosic material (i.e., without a previous chemical pretreatment)

attaining a lignin removal up to 37 %. The delignification can be improved up to 48 % if a subsequent alkaline peroxide extraction step is combined with the enzymatic treatment. Although these lignin removal values were attained with high mediator doses, noteworthy was the lignin reduction reached (up to 38 %) in lower-dose treatments, which would be more industrially attractive. In all cases assayed, the pretreated wheat straw was hydrolyzed with higher efficiency than the untreated material releasing higher yields of glucose and xylose by using relatively low doses of cellulases. The 2D NMR spectra of whole straw samples (at the gel stage) showed the selective action of laccase-mediator on the lignin moiety, while the polysaccharide signals remained unchanged with respect to the controls. This included the breakdown of inter-unit linkages and removal of lignin S and G units, although tricin remained in the residual lignin. The presence of oxidized S and G lignin units in the pretreated wheat straw provides evidence for a C_α-oxidation mechanism (and β-*O*-4′ cleavage) of lignin degradation even in the treatments with laccase alone. It should be pointed out that although promising results have been obtained in the present work with the laccase-mediator pretreatment of wheat straw, further optimization of the treatment including the increase in solid loading and the decrease of the laccase-mediator doses should be necessary to approach industrial conditions.

Acknowledgments This study was funded by the INDOX EU-project (KBBE-2013-7-613549); the LIGNOCELL, LIGNIN, NOESIS, and BIORENZYMERY Spanish MICINN (co-financed by FEDER funds) projects (AGL2011-25379, CTQ2014-60764-JIN, BIO2014-56388 R and AGL2014-53730-R); and the CSIC (201440E097) Project. A.P. thanks the Spanish MINECO for a FPI fellowship. A. Lomascolo and E. Record from INRA (Marseille, France) are acknowledged for the *P. cinnabarinus* laccase, and H. Lund and M. Tovborg from Novozymes (Bagsvaerd, Denmark) for Celluclast 1.5L and Novozyme 188.. The authors thank Dr. Angulo for performing the NMR analyses that were acquired on a Bruker AVANCE III 500-MHz instrument from the NMR facilities of the General Research Services of the University of Seville (SGI CITIUS).

References

1. Kim S, Dale BE (2004) Global potential bioethanol production from wasted crops and crop residues. Biomass Bioenergy 26: 361–375
2. Papa G, Varanasi P, Sun L, Cheng G, Stavila V, Holmes B, Simmons BA, Adani F, Singh S (2012) Exploring the effect of different plant lignin content and composition on ionic liquid pretreatment efficiency and enzymatic saccharification of Eucalyptus globulus L. mutants. Bioresource Technol 117:352–359
3. Studer MH, DeMartini JD, Davis MF, Sykes RW, Davison B, Keller M, Tuskan GA, Wyman CE (2011) Lignin content in natural *Populus variants* affects sugar release. Proc Natl Acad Sci U S A 108:6300–6305
4. Li X, Ximenes E, Kim Y, Slininger M, Meilan R, Ladisch M, Chapple C (2010) Lignin monomer composition affects Arabidopsis cell-wall degradability after liquid hot water pretreatment. Biotechnol Biofuels 3:27
5. Pareek N, Gillgren T, Jönsson LJ (2013) Adsorption of proteins involved in hydrolysis of lignocellulose on lignins and hemicelluloses. Bioresource Technol 148:70–77
6. Martínez AT, Ruiz-Dueñas FJ, Martínez MJ, del Río JC, Gutiérrez A (2009) Enzymatic delignification of plant cell wall: from nature to mill. Curr Opin Biotechnol 20:348–357
7. Xu F (2005) Applications of oxidoreductases: recent progress. Ind Biotechnol 1:38–50
8. Lai Y-Z (1992) Determination of phenolic hydroxyl groups. In: Lin SY, Dence CW (eds) Methods in lignin chemistry. Springer-Verlag, Berlin, pp 423–434
9. Bourbonnais R, Paice MG (1990) Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. FEBS Lett 267:99–102
10. Call H-P (1994) Verfahren zur Veränderung, Abbau oder Bleichen von Lignin, ligninhaltigen Materialien oder ähnlichen Stoffen. Patent (International) WO 94/29510
11. Poppius-Levlin K, Wang W, Tamminen T, Hortling B, Viikari L, Niku-Paavola M-L (1999) Effects of laccase/HBT treatment on pulp and lignin structures. J Pulp Pap Sci 25:90–94
12. Camarero S, García O, Vidal T, Colom J, del Río JC, Gutiérrez A, Gras JM, Monje R, Martínez MJ, Martínez AT (2004) Efficient bleaching of non-wood high-quality paper pulp using laccase-mediator system. Enzym Microb Technol 35:113–120
13. Gutiérrez A, del Río JC, Martínez AT (2009) Microbial and enzymatic control of pitch in the pulp and paper industry. Appl Microbiol Biotechnol 82:1005–1018
14. Prasetyo EN, Kudanga T, Ostergaard L, Rencoret J, Gutiérrez A, del Río JC, Santos JI, Nieto L, Jimenez-Barbero J, Martínez AT et al (2010) Polymerization of lignosulfonates by the laccase-HBT (1-hydroxybenzotriazole) system improves dispersibility. Bioresource Technol 101:5054–5062
15. Widsten P, Kandelbauer A (2008) Laccase applications in the forest products industry: a review. Enzym Microb Technol 42:293–307
16. Palonen H, Viikari L (2004) Role of oxidative enzymatic treatments on enzymatic hydrolysis of softwood. Biotechnol Bioeng 86:550–557
17. Heap L, Green A, Brown D, van Dongen B, Turner N (2014) Role of laccase as an enzymatic pretreatment method to improve lignocellulosic saccharification. Catal Sci Technol 4:2251–2259
18. Moilanen U, Kellock M, Vamai A, Andberg M, Viikari L (2014) Mechanisms of laccase-mediator treatments improving the enzymatic hydrolysis of pre-treated spruce. Biotechnol Biofuels 7:177
19. Gutiérrez A, Rencoret J, Cadena EM, Rico A, Barth D, del Río JC, Martínez AT (2012) Demonstration of laccase-mediator removal of lignin from wood and non-wood plant feedstocks. Bioresource Technol 119:114–122
20. Rico A, Rencoret J, del Río JC, Martínez AT, Gutiérrez A (2015) In-depth 2D NMR study of lignin modification during pretreatment of *Eucalyptus* wood with laccase and mediators. Bioenergy Res 8: 211–230
21. Chen Q, Marshall MN, Geib SM, Tien M, Richard TL (2012) Effects of laccase on lignin depolymerization and enzymatic hydrolysis of ensiled corn stover. Bioresource Technol 117:186–192
22. Jurado M, Prieto A, Martínez-Alcalá MA, Martínez AT, Martínez MJ (2009) Laccase detoxification of steam-exploded wheat straw for second generation bioethanol. Bioresource Technol 100:6378–6384
23. Oliva-Taravilla A, Tomás-Pejó E, Demuez M, Gonzalez-Fernandez C, Ballesteros M (2015) Inhibition of cellulose enzymatic hydrolysis by laccase-derived compounds from phenols. Biotechnol Prog 31:700–706

24. Herpoël I, Moukha S, Lesage-Meessen L, Sigoillot JC, Asther M (2000) Selection of *Pycnoporus cinnabarinus* strains for laccase production. FEMS Microbiol Lett 183:301–306
25. Bourbonnais R, Paice MG, Freiermuth B, Bodie E, Borneman S (1997) Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. Appl Environ Microbiol 63: 4627–4632
26. Rencoret J, Marques G, Gutiérrez A, Nieto L, Santos I, Jiménez-Barbero J, Martínez AT, del Río JC (2009) HSQC-NMR analysis of lignin in woody (*Eucalyptus globulus* and *Picea abies*) and non-woody (*Agave sisalana*) ball-milled plant materials at the gel state. Holzforschung 63:691–698
27. Kim H, Ralph J, Akiyama T (2008) Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d_6. Bioenergy Res 1:56–66
28. Seo DJ, Fujita H, Sakoda A (2011) Structural changes of lignocelluloses by a nonionic surfactant, Tween 20, and their effects on cellulase adsorption and saccharification. Bioresource Technol 102:9605–9612
29. Babot ED, Rico A, Rencoret J, Kalum L, Lund H, Romero J, del Río JC, Martínez AT, Gutiérrez A (2011) Towards industrially feasible delignification and pitch removal by treating paper pulp with *Myceliophthora thermophila l*accase and a phenolic mediator. Bioresource Technol 102:6717–6722
30. Tappi (2006) 2006–2007 TAPPI test methods. Norcoss, GA 30092, USA: TAPPI Press
31. Selvendran RR, March JF, Ring SG (1979) Determination of aldoses and uronic acid content of vegetable fiber. Anal Biochem 96: 282–292
32. del Río JC, Rencoret J, Prinsen P, Martínez AT, Ralph J, Gutiérrez A (2012) Structural characterization of wheat straw lignin as revealed by analytical pyrolysis, 2D-NMR, and reductive cleavage methods. J Agric Food Chem 60:5922–5935
33. Hatakka A, Hammel KE (2010) Fungal biodegradation of lignocelluloses. In: Hofrichter M (ed) The Mycota. Industrial applications, vol 10. Springer-Verlag, Berlin, pp 319–340
34. del Río JC, Prinsen P, Rencoret J, Nieto L, Jiménez-Barbero J, Ralph J, Martínez AT, Gutiérrez A (2012) Structural characterization of the lignin in the cortex and pith of Elephant grass (*Pennisetum purpureum*) stems. J Agric Food Chem 60:3619–3634
35. Moilanen U, Kellock M, Galkin S, Viikari L (2011) The laccase-catalyzed modification of lignin for enzymatic hydrolysis. Enzym Microb Technol 49:492–498
36. Ehara K, Tsutsumi Y, Nishida T (2000) Role of Tween 80 in biobleaching of unbleached hardwood kraft pulp with manganese peroxidase. J Wood Sci 46:137–142
37. Rico A, Rencoret J, del Río JC, Martínez AT, Gutiérrez A (2014) In-depth 2D NMR study of lignin modification during pretreatment of *Eucalyptus* wood with laccase and mediators. Bioenergy Res 8: 211–230
38. Rico A, Rencoret J, del Río JC, Martínez AT, Gutiérrez A (2014) Pretreatment with laccase and a phenolic mediator degrades lignin and enhances saccharification of *Eucalyptus* feedstock. Biotechnol Biofuels 7:6
39. Prinsen P, Gutiérrez A, Rencoret J, Nieto L, Jiménez-Barbero J, Burnet A, Petit-Conil M, Colodette JL, Martínez AT, del Río JC (2012) Morphological characteristics and composition of lipophilic extractives and lignin in Brazilian woods from different eucalypt hybrids. Ind Crops Prod 36:572–583
40. Rencoret J, Marques G, Gutiérrez A, Ibarra D, Li J, Gellerstedt G, Santos JI, Jiménez-Barbero J, Martínez AT, del Río JC (2008) Structural characterization of milled wood lignin from different eucalypt species. Holzforschung 62:514–526
41. Moreno AD, Ibarra D, Alvira P, Tomás-Pejó E, Ballesteros M (2015) Exploring laccase and mediators behavior during saccharification and fermentation of steam-exploded wheat straw for bioethanol production. J Chem Technol Biotechnol. doi:10.1002/jctb.4774
42. Alvira P, Negro MJ, Ballesteros I, González A, Ballesteros M (2016) Steam explosion for wheat straw pretreatment for sugars production. Bioethanol 2:66–75
43. Kirk TK, Farrell RL (1987) Enzymatic "combustion": the microbial degradation of lignin. Annu Rev Microbiol 41:465–505
44. Ruiz-Dueñas FJ, Martínez AT (2009) Microbial degradation of lignin: how a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this. Microb Biotechnol 2:164–177
45. Kawai S, Nakagawa M, Ohashi H (2002) Degradation mechanisms of a nonphenolic β-O-4 lignin model dimer by *Trametes versicolor* laccase in the presence of 1-hydroxybenzotriazole. Enzym Microb Technol 30:482–489
46. Cantarella G, Galli C, Gentili P (2003) Free radical versus electron-transfer routes of oxidation of hydrocarbons by laccase-mediator systems. Catalytic and stoichiometric procedures. J Mol Catal B-Enzym 22:135–144
47. Areskogh D, Li J, Nousiainen P, Gellerstedt G, Sipila J, Henriksson G (2010) Oxidative polymerisation of models for phenolic lignin end-groups by laccase. Holzforschung 64:21–34
48. Eriksson K-EL, Blanchette RA, Ander P (1990) Microbial and enzymatic degradation of wood components. Springer-Verlag, Berlin
49. Gierer J, Ljunggren S (1979) Reactions of lignins during sulfate pulping.16. Kinetics of the cleavage of β-aryl ether linkages in structures containing carbonyl groups. Svensk Papperstidning-Nordisk Cellulosa 82:71–81

PUBLICACIÓN 2:

Rencoret J., Pereira A., del Río J.C., Martínez A.T., Gutiérrez A. (2017) Delignification and saccharification enhancement of sugarcane byproducts by a laccase-based pretreatment. *ACS Sustainable Chemistry & Engineering* 5: 7145-7154.

ACS Sustainable Chemistry & Engineering

Research Article

pubs.acs.org/journal/ascecg

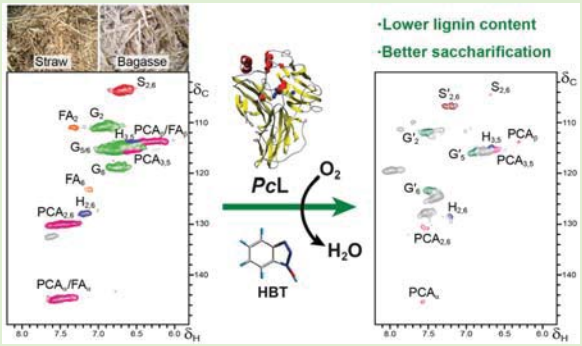
Delignification and Saccharification Enhancement of Sugarcane Byproducts by a Laccase-Based Pretreatment

Jorge Rencoret,*[,†] Antonio Pereira,[†] José C. del Río,[†] Ángel T. Martínez,[‡] and Ana Gutiérrez[†]

[†]Instituto de Recursos Naturales y Agrobiología de Sevilla, CSIC, Reina Mercedes 10, E-41012 Seville, Spain

[‡]Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, E-28040 Madrid, Spain

ABSTRACT: Sugarcane bagasse and straw, two major agro-industrial byproducts generated by the sugarcane industry, contain significant amounts of carbohydrates that can be hydrolyzed and then converted into ethanol or other valuable compounds. However, access to them is limited by the presence of lignin, a recalcitrant polymer that protects cell-wall polysaccharides from enzymatic hydrolysis. This work demonstrates the ability of an enzymatic pretreatment, based on the laccase from *Pycnoporus cinnabarinus*, and 1-hydroxybenzotriazole as mediator, to remove and/or modify lignin in sugarcane bagasse and straw residues, improving their subsequent saccharification. Up to 27% and 31% decreases of relative lignin content in ground sugarcane bagasse and straw, respectively, were achieved by the laccase-mediator pretreatment followed by alkaline peroxide extraction. Moreover, the lignin removal directly correlated with improvements in enzymatic saccharification, increasing glucose releases by around 39% and 46% for bagasse and straw, respectively, compared with those of the corresponding controls. Lignin depolymerization and degradation were made evident in the 2D-NMR spectra by a significant reduction in the number of aliphatic side chains involved in the main *β*-*O*-4′ and *β*-5′ interunit linkages, together with a remarkable removal of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) lignin units as well as the associated *p*-coumarates and ferulates, with respect to polysaccharides.



KEYWORDS: *Sugarcane residues, Bioethanol, Lignocellulose, Lignin removal, Oxidoreductases, Hydrolysis, 2D-NMR*

INTRODUCTION

In recent years, there has been an emerging effort to increase the production of biofuels from renewable feedstocks as a consequence of growing concerns about the depletion of fossil fuel reserves and global warming from the use of nonrenewable resources. In this context, lignocellulosic biomass represents a sustainable alternative platform to fossil resources since it is the most abundant and renewable biomass on Earth. Second-generation bioethanol, obtained after hydrolysis and fermentation of carbohydrates present in lignocellulosic materials, represents a real alternative to reduce the fossil fuel demand. Among the different lignocellulosic biomasses, agro-industrial lignocellulosic residues, such as sugarcane bagasse and straw, are attractive feedstocks for bioethanol production because of their high carbohydrate contents.[1] Sugarcane bagasse and straw are generated in significant amounts by the sugarcane industry,[2] around 125−140 kg[3] and 85−115 kg (dry weight),[4] respectively, per metric ton of sugarcane, and are readily available at low cost.

Carbohydrates are found in lignocellulosic materials as cellulose and hemicelluloses, two major structural polymers of the plant cell-wall together with lignin. The carbohydrate fraction, especially cellulose, can be converted into fermentable reducing sugars by enzymatic hydrolysis or chemical methods.[5] The hydrolysis is usually carried out by cellulolytic enzymes,[6] and the fermentation is accomplished by using yeasts[7] or bacteria.[8] However, the accessibility of the hydrolytic enzymes to the carbohydrates in the lignocellulosic materials is hampered, to a certain extent, by the presence of lignin. Lignin is an amorphous, cross-linked, and complex aromatic polymer consisting of mainly three different phenyl-propane units linked together by different ether and carbon−carbon bonds.[9] Lignin acts as a biological cement contributing to the formation of a highly recalcitrant lignocellulosic matrix. Lignin has been shown to have a negative effect on the enzymatic hydrolysis of cell-wall polysaccharides because it strongly reduces the access of enzymes[10] and also binds to them thus reducing their activity.[11] Hence, a biomass pretreatment step is needed to partially remove lignin and break down the lignocellulose structure, making cellulose more accessible during subsequent saccharification. Given the potential of sugarcane residues for the production of second-generation bioethanol, many pretreatment processes have been developed to reduce their recalcitrance to enzymatic hydrolysis.[12] These include, among others, steam explosion,[13] organosolv,[14] liquid hot water,[15]

Received: April 28, 2017
Revised: June 8, 2017
Published: July 4, 2017

DOI: 10.1021/acssuschemeng.7b01332
ACS Sustainable Chem. Eng. 2017, 5, 7145−7154

ammonia fiber explosion,[16] wet oxidation,[17] alkali delignification, and dilute acid hydrolysis.[18]

Biotechnology based on lignin-degrading microbes and their enzymes can contribute to the efficient and ecofriendly use of lignocellulosic feedstocks for the sustainable production of bioethanol.[19] In nature, efficient and selective lignin biodegradation is mediated mainly by white-rot fungi and certain bacteria.[20,21] The ligninolytic enzymes involved in this process are classified as peroxidases (lignin, manganese, and versatile peroxidases) and laccases.[20] Among these, laccases (phenoloxidases, EC 1.10.3.2) seem to be the most suitable enzymes for industrial applications as they can be produced on a large scale,[22] have broad substrate specificity, and utilize atmospheric oxygen as an electron acceptor to produce water. Several laccases have been shown to be capable of degrading different types of lignin, including natural[23−26] and synthetic (dehydrogenation polymer, DHP) lignins.[27] They oxidize either the minor phenolic components of lignin (less than 20%) directly (by themselves) or the phenolic and nonphenolic components, in the presence of a proper redox mediator, indirectly. As a result, radicals are generated in lignin, which can lead to bond cleavage and, consequently, to lignin depolymerization.

However, little is known about the application of biological pretreatments on sugarcane residues, and almost all of them are limited to the use of lignin-degrading fungi.[28−30] These fungal pretreatments present certain advantages over the thermochemical ones, such as mild reaction conditions, higher product yields, and less energy demand; nevertheless, they require a long incubation period. This drawback can be overcome by directly using the oxidoreductase enzymes (laccases and/or peroxidases) secreted by these microorganisms for lignin degradation. The enzymatic treatments, in contrast to those that are fungal-based, require the use of previously milled lignocellulosic materials, to increase the contact surface between enzyme and substrate, although substrate sterilization is not necessary.

Several studies have demonstrated the potential of the laccase-mediator system as an efficient pretreatment to remove lignin from both woody and nonwoody plant feedstocks to enhance their subsequent saccharification.[23−26] In the present study, a laccase-mediator system composed of the high-redox-potential laccase from the basidiomycete *Pycnoporus cinnabarinus* and 1-hydroxybenzotriazole (HBT) as mediator was investigated as a pretreatment to remove and/or modify the lignins in the sugarcane bagasse and straw residues for improved saccharification. *P. cinnabarinus* is one of the fungi selected for the production of high-redox-potential laccases for applications in lignocellulose biorefineries and other industrial processes.[31,32] The secretion rate, over 1.5 g L^{-1} enzyme (in cultures containing 5 g L^{-1} fungal biomass), is among the highest reported for a natural laccase producing fungus, and similar levels have been attained during laccase heterologous expression. Interestingly, under optimized production conditions, laccase is by far the most abundant extracellular oxidoreductase secreted by *P. cinnabarinus*, and therefore, crude culture filtrates could be used for lignocellulose pretreatment, without enzyme purification, as suggested for other applications.[33]

EXPERIMENTAL SECTION

Lignocellulosic Feedstocks, Enzyme, and Mediator. Sugarcane bagasse and straw were supplied by a midsized ethanol mill located in Minas Gerais state, Brazil. Sugarcane plants were harvested at the age of 6−8 months from high-performance sugarcane (*Saccharum* sp. hybrids) plantations. The sugarcane plants were manually collected and cleaned in the field where about one third of their weight was removed in the form of tops and leaves; this material is known as sugarcane straw or trash by the sugarcane industry. Bagasse is the solid byproduct that remains after sugarcane stalks are crushed to extract the juice. Sugarcane bagasse and straw samples were air-dried and ground using an IKA knife mill and then finely milled using a Retsch PM100 mill at 400 rpm for 6 h (with 10 min breaks after every 10 min of milling).

A fungal laccase from the basidiomycete *P. cinnabarinus*, provided by INRA (Marseille, France), was used in this study. Its activity, measured as the initial rate during oxidation of 5 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), ABTS (Roche Diagnostics, Mannheim, Germany), to its cation radical (ε_{436} 29 300 M^{-1} cm^{-1}) in 0.1 M sodium acetate (pH 5) at 24 °C, was 525 U mL^{-1}; the activity unit (U) was defined as the amount of enzyme oxidizing 1 μmol of ABTS per min. HBT from Sigma-Aldrich (Steinheim, Germany) was used as the redox mediator.

Laccase-Mediator Treatments. The enzymatic pretreatment used to delignify the sugarcane bagasse and straw residues consisted of a sequence of four laccase-mediator treatments, using the *P. cinnabarinus* laccase and HBT as redox mediator, each one followed by an alkaline peroxide extraction step. These pretreatment conditions were found to efficiently remove lignin from other woody (eucalyptus) and nonwoody (elephant grass) feedstocks with important improvements in their subsequent saccharification.[23] The treatments were carried out in 200 mL pressurized bioreactors (Labomat, Mathis) placed inside a shaker bath, at 170 rpm and 50 °C, using 2 g (dry weight) of the whole biomass at 6% solid loading (w:w) in 50 mM sodium tartrate buffer (pH 4) under O_2 atmosphere (2 bar) for 24 h. After the enzymatic treatment, the samples were filtered and washed with 1 L of water. In a subsequent step, samples at 6% solid loading (w:w) were submitted to a peroxide alkaline extraction using 1% (w:w) NaOH and 3% (w:w) H_2O_2 (also referred to dry weight) at 80 °C during 90 min, followed by water washing.[25] Treatments with laccase (50 U g^{-1}) alone (without the mediator) and controls without the laccase or mediator, were also performed (followed in both cases by the corresponding alkaline peroxide extractions) for comparison. Duplicate experiments of these treatments (including control, laccase alone, and laccase-HBT) were performed to estimate the variability in biological replicates. A statistical analysis, based on ANOVA (one-way analysis of variance) and the Tukey HSD test, was carried out to compare the effects of the different enzymatic treatments on the lignin removal and on the release of glucose. Lignin content was determined as Klason lignin, in triplicate assays, according to Tappi Method T222 om-88.[34] The data from both biological and technical replicates were averaged.

Saccharification of Treated Sugarcane Bagasse and Straw. The enzymatically treated sugarcane samples were hydrolyzed with a cocktail containing commercial enzymes (from Novozymes, Bagsvaerd) with cellulase [Celluclast 1.5 L; 2 FPU g^{-1} (FPU, filter-paper unit)] and β-glucosidase (Novozym 188; 6 U g^{-1}) activities, at 1% solid loading in 3 mL of 100 mM sodium citrate (pH 5) for 72 h at 45 °C, in a shaker bath at 140 rpm. The released glucose was determined as alditol acetate by gas chromatography (GC).[35] An HP 5890 gas chromatograph (Hewlett-Packard, Hoofddorp, The Netherlands) equipped with a split−splitless injector and a flame ionization detector was used. The injector and detector temperatures were set at 225 and 250 °C, respectively. Samples were injected in the split mode (split ratio 10:1), using a capillary column Agilent J&W DB-225 (30 m × 0.25 mm i.d. and 0.15 μm film thickness) and helium as the carrier gas. The oven was temperature-programmed from 220 (held for 5 min) to 230 (held for 5 min) °C at 2 °C min^{-1}. Peaks were quantified by area, and glucose was used as a standard to elaborate calibration curves. The data from three replicates were averaged.

2D-NMR Spectroscopy. For gel-state NMR sample preparation, ∼70 mg of ball-milled treated sugarcane bagasse and straw samples were transferred into 5 mm NMR tubes, and swelled in 0.75 mL of deuterated dimethyl sulfoxide (DMSO-d_6), forming a gel inside the

DOI: 10.1021/acssuschemeng.7b01332
ACS Sustainable Chem. Eng. 2017, 5, 7145−7154

NMR tube.[36,37] Heteronuclear single quantum coherence (HSQC) 2D-NMR experiments were run at 298 K on a Bruker AVANCE III 500 MHz spectrometer fitted with a 5 mm TCI (triple cryoprobe inverse) probe. The 2D-HSQC spectra were acquired using an adiabatic pulse sequence (Bruker standard pulse program "hsqcetgpsisp.2"), which enabled a semiquantitative analysis of the different $^{13}C-^{1}H$ correlation signals.[38] Spectra were acquired from 10 to 0 ppm in F2 (^{1}H) using 1000 data points for an acquisition time (AQ) of 100 ms, an interscan delay (D1) of 1 s, and from 200 to 0 ppm in F1 (^{13}C) using 256 increments of 32 scans, for a total acquisition time of 2 h 34 min. The $^{1}J_{CH}$ used was 145 Hz. Processing used typically matched Gaussian apodization in ^{1}H (parameters LB = −0.1 and GB = 0.001) and a squared cosine bell in ^{13}C (LB = 0.3 and GB = 0.1). The central residual DMSO peak (δ_C/δ_H, 39.5/2.49) was used as an internal reference. The $^{13}C-^{1}H$ correlation signals from the aromatic/unsaturated region of the spectrum were used to estimate the content of lignin, *p*-coumarate, and ferulate, and the lignin composition in terms of H, G, S, and oxidized S (S′) and G (G′) units. The amorphous polysaccharide content was estimated by the integration of xylose and glucose anomeric signals, whereas the correlation signals in the aliphatic oxygenated region were used to determine the interunit linkage and end-unit abundances in lignin.[26]

RESULTS AND DISCUSSION

In this work, we evaluate for the first time the ability of a laccase-based enzymatic pretreatment to successfully delignify and improve the saccharification of nonchemically pretreated sugarcane residues (bagasse and straw), which present rather different lignin compositions. Bagasse is enriched in S-lignin units and uncondensed *β*-ether linkages, whereas straw is enriched in G-lignin units and presents more condensed linkages.[1] Furthermore, the structural modifications produced in the lignin polymer of these sugarcane byproducts during the enzymatic treatment were analyzed in situ by two-dimensional nuclear magnetic resonance (2D-NMR) at the gel state,[36,37] which provided useful information regarding the lignin degradation mechanism.

Delignification of Sugarcane Bagasse and Straw with Laccase-HBT. The lignin contents (as Klason lignin) of the sugarcane bagasse and straw samples after the laccase-mediator pretreatment were determined and compared with their corresponding controls (Table 1). The lignin contents in the control samples that were processed as the full enzymatic treatment but without the presence of laccases and mediators were not modified with respect to the initial sugarcane residues. The treatments with laccase alone only decreased the lignin content of bagasse by about 4% (relative to the control), whereas a more pronounced decrease of up to 9% of the lignin content was attained in the case of straw. This low extent of degradation with laccase alone is in agreement with previous works using other lignocellulosic materials.[23−26] It is known that laccases alone are not very efficient for degrading the lignin in lignocellulosic materials because of the steric hindrance caused by their bulky molecular size and also because of their relatively low oxidation potentials, which only allow them to oxidize the minor phenolic hydroxyl groups present in lignin. However, it is important to highlight the higher extent of lignin degradation in the straw (nearly 2-fold higher), although straw only has a slightly lower lignin content than bagasse. This fact seems to suggest that the lignin composition of these materials, which is different in bagasse (S-rich lignin) and straw (G-rich lignin),[1] plays a major role in the lignin degradation with laccase alone. Previous works have shown that G-lignin units are present as phenolic units in a higher extent than the S-lignin units, which are mostly etherified,[39,40] and this would explain

Table 1. Percentage of the Initial Material Recovered, Lignin Content, Glucose Released by Enzymatic Hydrolysis, and in Vitro Digestibility of Sugarcane Residue Samples[a]

sample	recovery (%)	lignin (%)[b]	glucose (%)[b]	digestibility (%)[c]
Sugarcane Bagasse				
initial sugarcane bagasse		17.8 ± 0.6	35.9 ± 0.7	71.7 ± 1.1
control[d]	88.5	17.5 ± 0.4a	40.1 ± 0.2a	70.9 ± 0.3a
laccase (50 U g^{-1})[d]	80.5	16.8 ± 0.3a	44.2 ± 0.4b	71.3 ± 0.3a
laccase (50 U g^{-1}), HBT (3%)[d]	74.5	12.8 ± 0.3b	55.8 ± 0.4c	82.9 ± 0.6b
Sugarcane Straw				
initial sugarcane straw		17.0 ± 0.2	34.9 ± 0.2	75.4 ± 0.4
control[d]	90.5	16.6 ± 0.2a	39.2 ± 0.2a	76.5 ± 0.4a
laccase (50 U g^{-1})[d]	82.0	15.1 ± 0.1b	42.3 ± 0.1b	75.0 ± 0.2a
laccase (50 U g^{-1}), HBT (3%)[d]	75.5	11.5 ± 0.3c	57.1 ± 0.3c	93.0 ± 0.5b

[a]Means ± SD (standard deviation) shown were obtained from technical triplicates. Letters next to the SD, from the Tukey test, show results not significantly different from the control (a), significantly different from the control (b) and significantly different from both the control and the laccase-alone results, at the 0.05 level. [b]Values for the lignin content (expressed as Klason lignin) and glucose released after cellulase hydrolysis of samples treated with *P. cinnabarinus* laccase (50 U g^{-1}) and HBT (3%), followed by an alkaline peroxide extraction (Ep), are compared with values for a control without enzyme, a treatment only with laccase, and the initial sugarcane bagasse and straw samples. [c]Digestibility values were calculated on the basis of the total glucan content in the initial sugarcane residues and the percentages of released glucose, with consideration in each case for the percentage of recovery. [d]Enzymatic/Ep pretreated (4 cycles).

their higher degradation extent with laccases alone. However, the lignin content in both lignocellulosic residues significantly decreased after the enzymatic treatment using the laccase-HBT system. For sugarcane bagasse, the lignin reduction was about 27% of the initial lignin content whereas in the case of sugarcane straw up to 31% of lignin removal was achieved. Similar delignification degrees (ca. 32%) were obtained with elephant grass using the same enzymatic conditions, although much higher delignification degrees (nearly 50% lignin removal) could be attained with eucalyptus wood.[23] The differences in the delignification extents of elephant grass and those of eucalyptus wood were attributed to the predominance of syringyl lignin units in the latter.[23] Nevertheless, this seems not to be the case for the sugarcane residues selected for this study since sugarcane bagasse is enriched in S-lignin units[1] but presented a slightly lower delignification extent than the straw, which is enriched in G-lignin units[1] and presented a slightly higher delignification extent. These data indicate that, in the pretreatment of sugarcane residues, other structural features of the lignin polymer beyond its composition (H:G:S ratio) have to be considered to explain the obtained delignification results. In this sense, a possible reason could be related to the presence of *p*-coumarates acylating the *γ*-OH of the lignin side chains, which could hamper in some way the action of laccases. This fact would explain why sugarcane straw, with a percentage of *p*-coumarates of 47% (referred to lignin), is delignified to a greater extent than sugarcane bagasse, which has a higher content of *p*-coumarates (77%). This hypothesis would also

DOI: 10.1021/acssuschemeng.7b01332
ACS Sustainable Chem. Eng. 2017, 5, 7145−7154

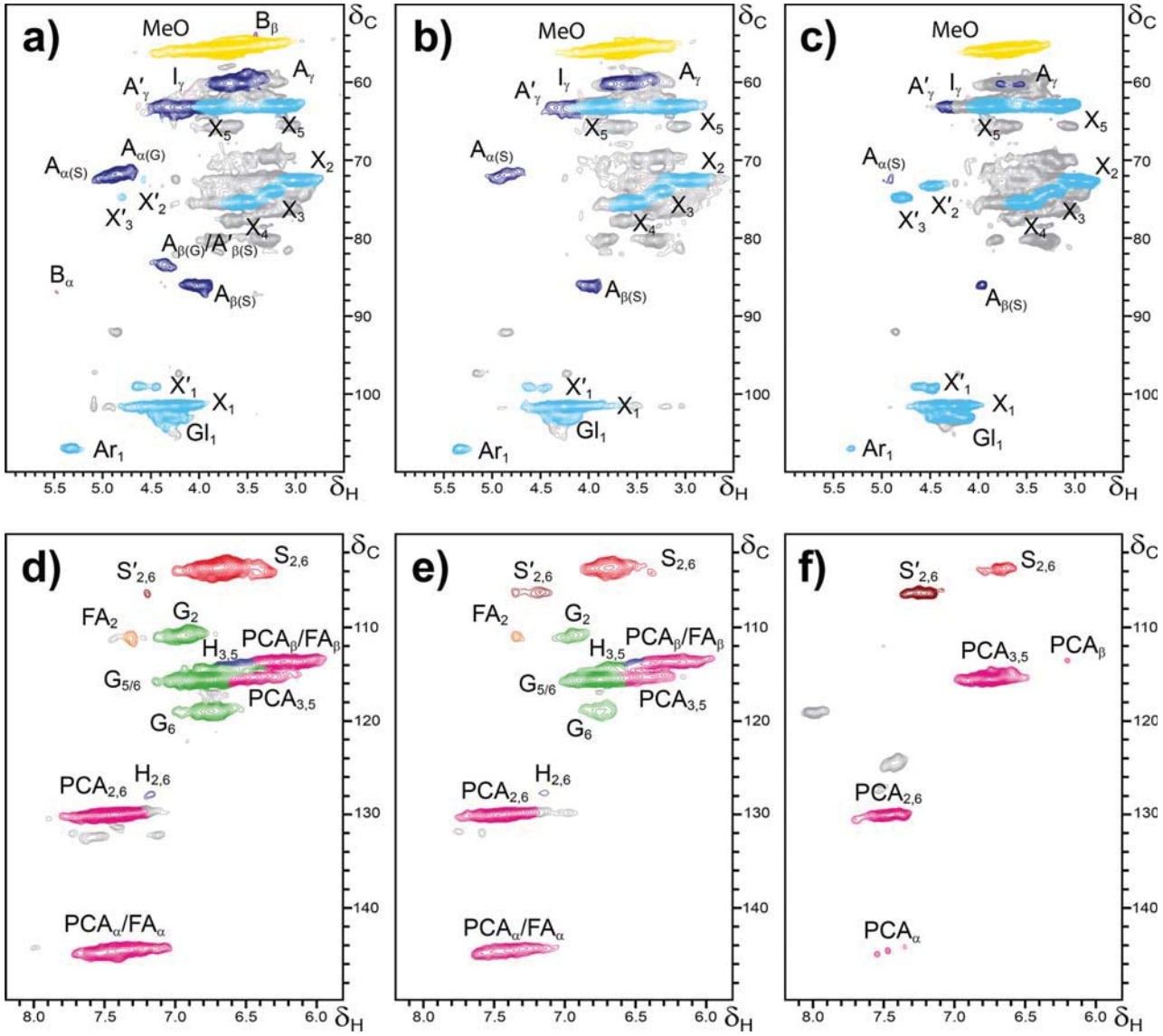


Figure 1. HSQC NMR spectra of sugarcane bagasse after laccase-mediator treatment and a subsequent alkaline peroxide extraction (4 cycles). Expanded aliphatic oxygenated (δ_H/δ_C, 2.5−6.0 and 50−110 ppm; top) and aromatic (δ_H/δ_C, 5.8−8.2 and 90−150 ppm; bottom) regions of the HSQC NMR spectra of sugarcane bagasse treated with *P. cinnabarinus* laccase-HBT: (a, d) control without enzyme, (b, e) 50 U g^{-1} enzyme, and (c, f) 50 U g^{-1} enzyme and 3% HBT. See Table 2 for lignin signal assignments, Figure 3 for the main lignin structures identified, and Table 3 for quantification of these lignin structures. Carbohydrate signals are also observed mainly corresponding to C_1−C_5 in normal (X_1−X_5) and acetylated (X'_1−X'_5) xylan units (anomeric glucose and arabinose signals were also identified: G_1 and Ar_1). Unassigned signals are in gray, including signals from the enzyme and the mediator.

explain that the lignin removal attained after pretreatment of elephant grass (32%)[23] was close to those achieved in the case of sugarcane bagasse and straw pretreatment since elephant grass lignin is also γ-*p*-coumaroylated (~40%).[41] Finally, in the case of eucalypt wood, whose lignin is not acylated at the γ-OH of the side chains,[42] the pretreatment should be more effective, and this would explain why the attained lignin removal was nearly 50%.

Enzymatic Hydrolysis of Sugarcane Bagasse and Straw Pretreated with Laccase-HBT. The sugarcane bagasse and straw samples treated with laccase-HBT and those treated with laccase alone (without mediator), as well as the control samples, were hydrolyzed (72 h) using low doses of cellulases (2 FPU g^{-1}) and β-glucosidase (6 U g^{-1}),[23] and the released glucose was quantified as alditol acetate by GC. The saccharification results (Table 1) indicate a direct correlation between the lignin removal and the increase in glucose yield obtained by enzymatic hydrolysis. After the treatment with laccase-HBT, relative glucose releases improved up to 39% and 46%, for bagasse and straw, respectively, with respect to the control samples. Interestingly, enzymatic treatments with laccase alone also showed a positive effect in the subsequent enzymatic saccharification, with improvements in glucose release around 10% for both bagasse and straw. This positive effect of the laccase alone has also been observed during the enzymatic pretreatment of wheat straw,[26] and is attributed to both the delignification of the phenolic lignin moiety and the reduction of the nonspecific bindings of cellulases to lignin,

DOI: 10.1021/acssuschemeng.7b01332
ACS Sustainable Chem. Eng. 2017, 5, 7145−7154

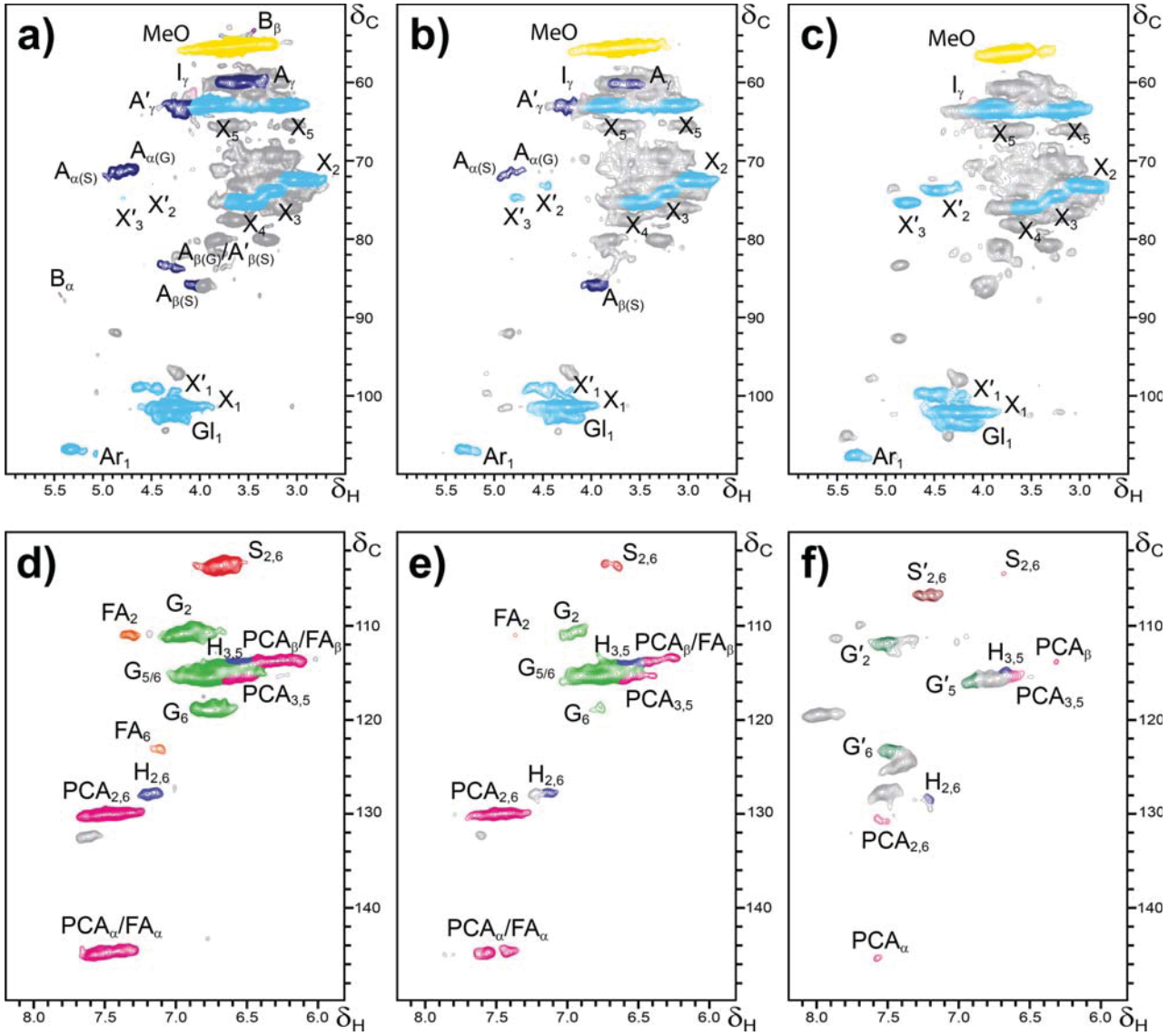


Figure 2. HSQC NMR spectra of sugarcane straw after laccase-mediator treatment and a subsequent alkaline peroxide extraction (4 cycles). Expanded aliphatic oxygenated (top) and aromatic (bottom) regions of the HSQC NMR spectra of sugarcane straw samples after treatment with laccase-HBT followed by an alkaline peroxide extraction: (a, d) control without enzyme, (b, e) 50 U g^{-1} enzyme, and (c, f) 50 U g^{-1} enzyme and 3% HBT. See the Figure 1 legend for additional information.

which make them inactive.[43] Moreover, a treatment with laccase alone could also modify the lignocellulosic substrate surface by increasing the amounts of carboxylic acids, reducing in this way the nonspecific adsorption of negatively charged cellulases.[44] A possibility for the industrial implementation and environmental feasibility of the laccase pretreatments would be the use of natural phenolic mediators instead of synthetic mediators,[45] as already proposed for paper pulp delignification.[46]

2D-NMR Analysis of Sugarcane Bagasse and Straw after Laccase-HBT Pretreatment. The main structural modifications produced in sugarcane bagasse and straw by the laccase-mediator treatment were studied by 2D-NMR of whole cell-walls at the gel state, according to the previously developed methodology.[36,37] This technique allows for the in situ structural characterization of lignins, without the need of prior lignin isolation that sometimes results in alterations of the lignin polymer structure, and carbohydrates in the cell-wall. Finely ball-milled samples were swelled in DMSO-d_6 to form a gel inside the NMR tube and then were analyzed by HSQC 2D-NMR experiments.[36,37] The spectra of enzymatically pretreated (with laccase-HBT and laccase alone) sugarcane bagasse and straw samples (including the respective control samples) are shown in Figures 1 and 2. The aliphatic oxygenated region of the spectra (δ_H/δ_C, 2.5−6.0 and 50−110) shows correlation signals from lignin (side chains and methoxyl groups) and carbohydrates (mainly from hemicelluloses and noncrystalline cellulose), whereas the aromatic/unsaturated region of the spectra (δ_H/δ_C, 5.8−8.2 and 90−150) includes signals from lignin units (H, G, S) as well as signals from the associated *p*-coumarates and ferulates. The lignin and carbohydrate cross-signals assigned in the HSQC spectra are listed in Table 2, and the main lignin units and substructures are depicted in Figure 3. The structural characteristics of the

DOI: 10.1021/acssuschemeng.7b01332
ACS Sustainable Chem. Eng. 2017, 5, 7145−7154

Table 2. Assignments of $^{13}C/^{1}H$ Correlation Signals in the 2D-HSQC Spectra from the Whole Cell-Walls of Treated Sugarcane Bagasse and Straw

label	δ_C/δ_H (ppm)	assignment
	Lignin Signals	
B_β	53.4/3.45	C_β/H_β in phenylcoumaran (B)
OCH_3	55.5/3.72	C/H in methoxyls
A_γ	59.6/3.37 and 3.71	C_γ/H_γ in γ-hydroxylated β-O-4′ (A)
I_γ	61.3/4.07	C_γ/H_γ in cinnamyl alcohol end-groups (I)
A'_γ	62.9/4.16 and 4.35	C_γ/H_γ in γ-acylated β-O-4′ (A′)
$A_{\alpha(G)}$	71.1/4.71	C_α/H_α in β-O-4′ (A) linked to a G unit
$A_{\alpha(S)}$	71.5/4.81	C_α/H_α in β-O-4′ (A) linked to an S unit
$A'_{\beta(S)}$	83.0/4.33	C_β/H_β in γ-acylated β-O-4′ linked (A′) to an S unit
$A_{\beta(G)}$	83.5/4.38	C_β/H_β in β-O-4′ linked (A) to a G unit
$A_{\beta(S)}$	85.9/4.09	C_β/H_β in β-O-4′ linked (A) to an S unit
B_α	87.0/5.48	C_α/H_α in phenylcoumaran (B)
$S_{2,6}$	103.8/6.69	C_2/H_2 and C_6/H_6 in etherified S units
$S'_{2,6}$	106.2/7.28 and 106.3/7.16	C_2/H_2 and C_6/H_6 in α-oxidized S′ units
G_2	110.7/6.96	C_2/H_2 in G units
FA_2	110.9/7.33	C_2/H_2 in ferulate
G'_2	111.4/7.50	C_2/H_2 in α-oxidized G′ units
PCA_β and FA_β	113.5/6.29	C_β/H_β in p-coumarate and ferulate
$H_{3,5}$	113.6/6.64	C_3/H_3 and C_5/H_5 in H units
G_5/G_6	114.9/6.78 and 6.94	C_5/H_5 and C_6/H_6 in G units
	118.8/6.77	
G'_5	115.0/6.73	C_5/H_5 in α-oxidized G′ units
$PCA_{3,5}$	115.3/6.76	C_3/H_3 and C_5/H_5 in p-coumarate
FA_6	123.2/7.12	C_6/H_6 in ferulate
$H_{2,6}$	127.8/7.19	C_2/H_2 and C_6/H_6 in H units
$PCA_{2,6}$	129.9/7.47	C_2/H_2 and C_6/H_6 in p-coumarate
PCA_α and FA_α	144.7/7.53	C_α/H_α in p-coumarate and ferulate
	Carbohydrate Signals	
X_5	63.0/3.16 and 3.87	C_5/H_5 in xylopyranose units
X_2	72.5/3.03	C_2/H_2 in xylopyranose units
X'_2	73.0/4.46	C_2/H_2 in 2-O-acetylated xylopyranose units
X_3	73.9/3.24	C_3/H_3 in xylopyranose units
X'_3	74.7/4.79	C_3/H_3 in 3-O-acetylated xylopyranose units
X_4	75.3/3.49	C_4/H_4 in xylopyranose units
X'_1	99.3/4.48	C_1/H_1 in 3-O-acetylated xylopyranose units
X_1/X'_1	101.5/4.26	C_1/H_1 in xylopyranose units
Gl_1	102.9/4.16	C_1/H_1 in glucopyranose units
Ar_1	107.0/5.33	C_1/H_1 in arabinofuranose units

lignins from the enzymatically treated sugarcane bagasse and straw, including the relative abundances of the different interunit linkages and cinnamyl end-groups, and the molar abundances of the lignin units (H, G, S, G′ and S′), p-coumarates, and ferulates, estimated from the volume integration of the signals in the HSQC spectra, are indicated in Table 3. In addition, the use of an adiabatic pulse sequence in the 2D-HSQC experiments allowed the estimation of the number of side chains per aromatic lignin unit, a parameter that provides valuable information regarding the lignin depolymerization.

Structural Modification of Sugarcane Bagasse. The expanded aliphatic oxygenated and aromatic/unsaturated regions of the HSQC spectra from the sugarcane bagasse samples are shown in Figure 1. The aliphatic oxygenated region of the spectrum of the control sample (Figure 1a) shows correlation signals of lignin and carbohydrates. Carbohydrate signals corresponded mainly to normal (nonacetylated, X) and acetylated (X′) xylans, whereas the most intense signals of lignin presented in this region corresponded to methoxyl groups (MeO) and β-O-4′ alkyl-aryl ether substructures (A), which represent up to 97% of all NMR-measurable (side chain) linkages in the control sample. Other signals corresponded to β-5′ phenylcoumaran substructures (B) and cinnamyl end-groups (I), but the latter were observed with low intensities in this region of the spectrum. The aromatic/unsaturated region of the HSQC spectra of the control sample (Figure 1d) shows that sugarcane bagasse has an S-rich lignin with an H/G/S molar composition of 1:37:61 (S/G ratio of 1.65). In addition, this lignin is associated with important amounts of p-coumarates and ferulates (77% and 6%, respectively, referring to the lignin content).

The HSQC spectrum of sugarcane bagasse after the enzymatic treatment with laccase alone (without mediator) is rather similar to that of the control sample, as expected from a lignin removal of only 4%, although the intensity of the signals of β-O-4′ alkyl-aryl ether substructures slightly decreased (Figure 1b). Likewise, the signals of the lignin units observed in the aromatic region (Figure 1e) showed a slight increase of Cα-oxidized S-lignin units ($S'_{2,6}$), confirming that laccase alone is able to oxidize the phenolic substructures presented in the lignin moiety, although only to a minor extent.

The HSQC spectrum of sugarcane bagasse treated with laccase and mediator (Figure 1c,f) revealed that the lignin polymer was largely degraded during the enzymatic pretreatment. While the correlation signals of carbohydrates remained mostly unchanged (with the exception of the signals X'_2 and X'_3 from acetylated xylans that increased probably because of the better mobility of these groups after removing lignin−carbohydrate linkages), most of the lignin correlation signals strongly decreased as compared to those of the control sample; hence, the signals for the β-O-4′ alkyl-aryl ether substructures (A), which were the most intense side-chain signals in the control sample, were barely detectable, and the β-5′ phenylcoumarans (B) were not detected. This is reflected in the semiquantitative NMR analysis that shows an important relative increase of sugar units from 71.4% (in the control sample) to 94.2% (after laccase-HBT treatment) (see Table 3). In addition, the aromatic region of the spectrum showed the complete removal of signals from H- and G-lignin units, as well as from ferulates, whereas only signals from S-lignin units and those from their associated p-coumarates, which are acylating the γ-OH,[1] remain after the laccase-mediator treatment. Similar results were also observed in the lignin of elephant grass after the enzymatic treatment with the same laccase-mediator system.[23] Furthermore, an important fraction of S units were Cα-oxidized as is evident from the relative increase in the $S'_{2,6}$ signals. The formation of oxidized lignin structures is in agreement with the nature of the lignin biodegradation process, described as an "enzymatic combustion".[47,48] Studies with nonphenolic lignin model compounds have reported that the laccase-HBT system first abstracts a hydrogen atom from the Cα position with the Cα-Cβ breakdown at a later stage.[49,50] The decrease of the total side chains (as per aromatic units), together with the increase of the minor cinnamyl end-groups, also confirms the depolymerization of the lignin.

DOI: 10.1021/acssuschemeng.7b01332
ACS Sustainable Chem. Eng. 2017, 5, 7145−7154

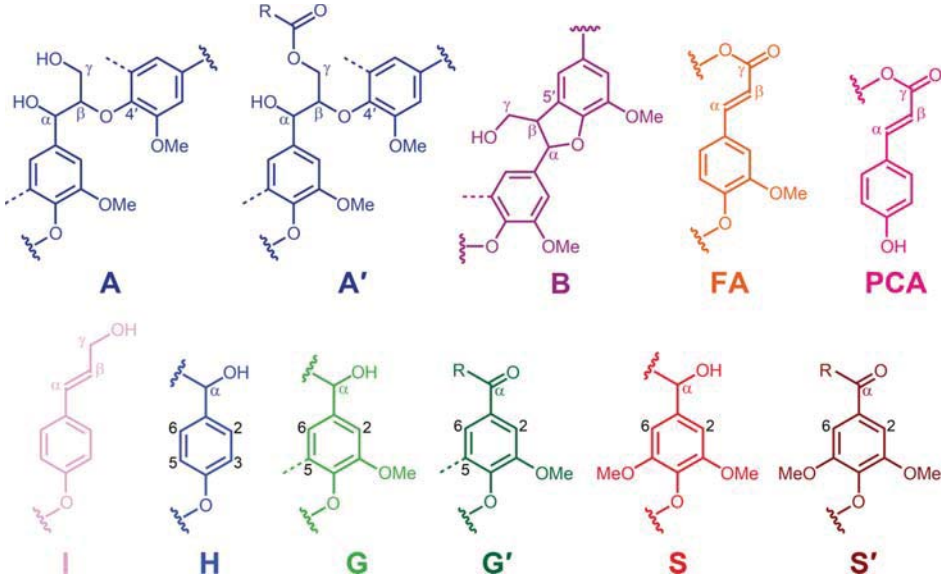


Figure 3. Main lignin units and substructures identified in the 2D-HSQC spectra of sugarcane bagasse and straw samples: (A) *β-O*-4′ alkyl-aryl ether structures, (A′) *β-O*-4′ structures with acylated *γ*-OH, (B) *β*-5′ phenylcoumaran structures, (FA) ferulate, (PCA) *p*-coumarate, (I) cinnamyl alcohol end-group, (H) *p*-hydroxyphenyl unit, (G) guaiacyl unit, (G′) C*α*-oxidized G unit, (S) syringyl unit, and (S′) C*α*-oxidized S unit. (R in G′ and S′ can be a hydroxyl in carboxylic acids, or a lignin side chain in ketones.)

Table 3. Semiquantitative NMR Analysis[a] of Sugarcane Bagasse and Straw Treated with Laccase (50 U g^{-1}) in Combination with HBT (3%) and Laccase Alone, Compared with Control Treatment (Without Enzyme)

	sugarcane bagasse			sugarcane straw		
	control	laccase	lac-HBT	control	laccase	lac-HBT
			Sample Composition[b]			
syringyl lignin units (S)	9.6 (61)	5.0 (59)	1.5 (50)	3.2 (25)	0.9 (17)	0 (1)
C*α*-oxidized S units (S′)	0.1 (1)	0.9 (11)	1.5 (50)	0 (0)	0.1 (1)	1.0 (40)
guaiacyl lignin units (G)	5.9 (37)	2.4 (29)	0 (0)	8.3 (66)	3.9 (71)	0 (0)
C*α*-oxidized G units (G′)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1.4 (52)
p-hydroxyphenyl units (H)	0.1 (1)	0.1 (1)	0 (0)	1.1 (9)	0.6 (10)	0.2 (7)
total lignin	15.7 (100)	8.4 (100)	3.0 (100)	12.6 (100)	5.4 (100)	2.6 (100)
p-coumarates (PCA)	12.0 (77)	8.4 (100)	2.8 (93)	5.9 (47)	4.1 (75)	0.2 (8)
ferulates (FA)	0.9 (6)	0.6 (8)	0 (0)	1.4 (11)	0.4 (8)	0 (0)
total aromatics	28.6	17.4	5.8	19.9	9.9	2.8
sugar units	71.4	82.6	94.2	80.1	90.1	97.2
total	100	100	100	100	100	100
lignin S/G ratio	1.66	2.43		0.38	0.31	0.79
			Side Chains and End-Groups[c]			
β-O-4′ ethers (A + A′)	97 (64)	97 (58)	84 (32)	89 (56)	90 (50)	0
phenylcoumarans (B)	1 (1)	0 (0)	0	4 (2)	0 (0)	0
cinnamyl end-groups (I)	2 (1)	3 (2)	16 (6)	7 (5)	10 (6)	100 (10)
total	100 (66)	100 (60)	100 (38)	100 (63)	100 (56)	100 (10)

[a]Values in parentheses refer to total lignin. [b]Sample composition presents the molar amount of normal (H, G, and S) and C*α*-oxidized (G′ and S′) lignin units, S/G ratio, *p*-coumarate (PCA), and ferulates (FA) on the basis of the integration of aromatic signals, and sugar units (mainly xylose and glucose) from the integration of anomeric carbon signals. [c]The percentages of lignin side chains involved in substructures A and B, and cinnamyl alcohol end-groups (I) are obtained from the integration of aliphatic signals in the HSQC spectra. Values in brackets refer to the total lignin units (H + G + G′+ S + S′).

Structural Modification of Sugarcane Straw. Contrary to that in sugarcane bagasse, the lignin in sugarcane straw is enriched in G-lignin units (H:G:S ratio of 9:66:25, S/G ratio of 0.38; for the control sample), and this is reflected in a different relative distribution of the main interunit linkages with respect to bagasse lignin (Table 3). Therefore, a different behavior toward laccase-mediator pretreatment might be expected.

The expanded aliphatic oxygenated and aromatic/unsaturated regions of the HSQC spectra from the sugarcane straw control and treated (with laccase alone and laccase-HBT) samples are shown in Figure 2. The aliphatic oxygenated region of the HSQC spectrum of the control sample showed correlation signals of both lignin and carbohydrates. As for what occurred in the bagasse spectrum, the main signals of carbohydrates corresponded to normal (nonacetylated; X_1, X_2,

DOI: 10.1021/acssuschemeng.7b01332
ACS Sustainable Chem. Eng. 2017, 5, 7145−7154

X_3, X_4, and X_5) and acetylated (X'_1 X'_2, and X'_3) xylans, as well as to arabinose (Ar_1) units. The most prominent signals of lignin present in this region corresponded to methoxyl groups (MeO) and β-*O*-4′ ethers (A), which represent up to 90% of all NMR-measurable linkages. β-5′ phenylcoumaran substructures (B), which require at least one guaiacyl unit to be formed, are also favored in G-rich lignin, and accounted for 4% of total linkages. The aromatic/unsaturated region of the HSQC spectrum of the control sample (Figure 2d) shows that sugarcane straw has a G-rich lignin, as mentioned above. Moreover, this lignin is associated with an important amount of *p*-coumarates and ferulates (47% and 11%, respectively, referring to the lignin content), as what also occurs in the bagasse lignin.

The enzymatic treatment of sugarcane straw with laccase alone produced a higher reduction in the intensity of the lignin signals, when compared to the same treatment on bagasse, in both the aliphatic oxigenated and the aromatic/unsaturated regions. The side-chain region of the spectrum (Figure 2b) showed a decrease of signals from β-*O*-4′ (A) and β-5′ phenylcoumaran (B) substructures, the latest of which completely disappeared from the spectrum. On the contrary, most of the carbohydrate signals remained unchanged, and even signals of acetylated xylan (X'_2 and X'_3) appeared, revealing that the cell-wall structure became altered in a way that some of the xylans increased their mobility. This relative increase of carbohydrates with respect to lignin is also reflected in the NMR semiquantitative analysis (Table 3), where carbohydrate anomeric signals increased from 80% (in the control sample) to 97% (after laccase-mediator treatment). In regards to the aromatic/unsaturated region of the spectrum (Figure 2e), the correlation signals of H-, G-, and S-lignin units, as well as the signals from *p*-coumarates and ferulates, clearly decreased with respect to the control, indicating a modification of the lignin structure by the action of the laccase alone. As mentioned above, laccases are able to degrade phenolic lignin, which is mostly related to G units,[32,33] and this would explain the higher overall lignin removal in the case of sugarcane straw.

The treatment of sugarcane straw with laccase-HBT produced considerable lignin removal (31% compared to the control sample, Table 1) that is reflected in the HSQC spectrum (Figure 2c,f). The aliphatic oxygenated region only showed signals from carbohydrates, which remained largely unchanged, while signals for lignin linkages completely disappeared from the spectrum. In the aromatic/unsaturated region of the spectrum, most of the lignin signals also disappeared, and only small signals from H- and S-lignin units, and from *p*-coumarates (acylating the γ-OH of S units), still remain. Signals for Cα-oxidized G- and S-lignin units (G′ and S′) now appeared in the spectrum, which were generated as a consequence of the oxidation produced by the laccase-mediator treatment. Interestingly, Cα-oxidized G units (G′), which were undetected in the spectrum of the control samples, were now easily observed, indicating the oxidation of the major G-lignin units present in sugarcane straw, as also observed in the laccase-HBT treatment of other G-enriched lignocellulosic materials, like wheat straw.[26]

CONCLUSIONS

Ground sugarcane bagasse and straw residues were partially delignified by the pretreatment with laccase-HBT, thus improving their subsequent enzymatic saccharification. The enzymatic pretreatment produced a rather similar lignin removal from both materials, despite their different lignin composition (H:G:S ratio), suggesting that other lignin structural features, as the presence of *p*-coumarates acylating the γ-OH of the side chains, can also play a role in the lignin removal process. The laccase-mediator treatment selectively acts on the lignin moiety, partially breaking down the interunit linkages and generating Cα-oxidized lignin units, whereas it leaves the carbohydrate fraction mostly unchanged, as indicated by 2D-NMR.

AUTHOR INFORMATION

Corresponding Author

*E-mail: jrencoret@irnase.csic.es.

ORCID

Jorge Rencoret: 0000-0003-2728-7331

Ángel T. Martínez: 0000-0002-1584-2863

Ana Gutiérrez: 0000-0002-8823-9029

Author Contributions

J.R. and A.P. contributed equally to this work. A.G. conceived the study and supervised the work. J.R. and A.P. carried out the experimental work. J.R. performed 2D NMR and analyzed the data. J.R., J.C.R., and A.G. wrote the manuscript. A.T.M. critically reviewed the manuscript with substantial contribution to its intellectual content. All authors read and approved the final manuscript.

Funding

This study has been funded by the Spanish projects AGL2014-53730-R and CTQ2014-60764-JIN (cofinanced by FEDER funds) and the EU-project INDOX (KBBE-2013-7-613549).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

A.P. thanks the Spanish MINECO for an FPI fellowship. Dr. Manuel Angulo is acknowledged for running the NMR experiments that were performed on a Bruker Avance III 500 MHz instrument from the NMR facilities of the General Research Services of the University of Seville (SGI CITIUS).

REFERENCES

(1) del Río, J. C.; Lino, A. G.; Colodette, J. L.; Lima, C. F.; Gutiérrez, A.; Martínez, Á. T.; Lu, F.; Ralph, J.; Rencoret, J. Differences in the chemical structure of the lignins from sugarcane bagasse and straw. *Biomass Bioenergy* **2015**, *81*, 322−338.

(2) Silva Ortiz, P.; de Oliveira, S., Jr Exergy analysis of pretreatment processes of bioethanol production based on sugarcane bagasse. *Energy* **2014**, *76*, 130−138.

(3) Molina, W. F., Jr.; Ripoli, T. C.; Geraldi, R. N.; do Amaral, J. R. Aspectos econômicos e operacionais do enfardamento de resíduos de colheita de cana-de-açúcar para aproveitamento energético. *STAB—Açúcar Álcool e Subprodutos* **1995**, *13*, 28−31.

(4) Singh, P.; Suman, A.; Tiwari, P.; Arya, N.; Gaur, A.; Shrivastava, A. K. Biological pretreatment of sugarcane trash for its conversion to fermentable sugars. *World J. Microbiol. Biotechnol.* **2008**, *24*, 667−673.

(5) Mosier, N.; Wyman, C.; Dale, B.; Elander, R.; Lee, Y. Y.; Holtzapple, M.; Ladisch, M. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour. Technol.* **2005**, *96* (6), 673−686.

(6) Chandra, M. S.; Viswanath, B.; Reddy, B. R. Cellulolytic enzymes on lignocellulosic substrates in solid state fermentation by *Aspergillus niger*. *Indian J. Microbiol.* **2007**, *47* (4), 323−328.

(7) Park, J. M.; Oh, B.-R.; Seo, J.-W.; Hong, W.-K.; Yu, A.; Sohn, J.-H.; Kim, C. H. Efficient production of ethanol from empty palm fruit bunch fibers by fed-batch simultaneous saccharification and

DOI: 10.1021/acssuschemeng.7b01332
ACS Sustainable Chem. Eng. 2017, 5, 7145−7154

fermentation using *Saccharomyces cerevisiae*. *Appl. Biochem. Biotechnol.* **2013**, *170* (8), 1807−1814.
(8) Ng, T. K.; Ben-Bassat, A.; Zeikus, J. G. Ethanol production by thermophilic bacteria: Fermentation of cellulosic substrates by cocultures of *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*. *Appl. Environ. Microbiol.* **1981**, *41* (6), 1337−1343.
(9) Vanholme, R.; Morreel, K.; Ralph, J.; Boerjan, W. Lignin biosynthesis and structure. *Plant Physiol.* **2010**, *153* (3), 895−905.
(10) Kumar, R.; Wyman, C. E. Does change in accessibility with conversion depend on both the substrate and pretreatment technology? *Bioresour. Technol.* **2009**, *100* (18), 4193−4202.
(11) Yarbrough, J. M.; Mittal, A.; Mansfield, E.; Taylor, L. E.; Hobdey, S. E.; Sammond, D. W.; Bomble, Y. J.; Crowley, M. F.; Decker, S. R.; Himmel, M. E.; Vinzant, T. B. New perspective on glycoside hydrolase binding to lignin from pretreated corn stover. *Biotechnol. Biofuels* **2015**, *8*, 214.
(12) Karp, S. G.; Woiciechowski, A. L.; Soccol, V. T.; Soccol, C. R. Pretreatment strategies for delignification of sugarcane bagasse: a review. *Braz. Arch. Biol. Technol.* **2013**, *56*, 679−689.
(13) Rocha, G. J. d. M.; Gonçalves, A. R.; Oliveira, B. R.; Olivares, E. G.; Rossell, C. E. V. Steam explosion pretreatment reproduction and alkaline delignification reactions performed on a pilot scale with sugarcane bagasse for bioethanol production. *Ind. Crops Prod.* **2012**, *35* (1), 274−279.
(14) Mesa, L.; González, E.; Cara, C.; González, M.; Castro, E.; Mussatto, S. I. The effect of organosolv pretreatment variables on enzymatic hydrolysis of sugarcane bagasse. *Chem. Eng. J.* **2011**, *168* (3), 1157−1162.
(15) Allen, S. G.; Kam, L. C.; Zemann, A. J.; Antal, M. J. Fractionation of sugarcane with hot, compressed, liquid water. *Ind. Eng. Chem. Res.* **1996**, *35* (8), 2709−2715.
(16) Krishnan, C.; Sousa, L. d. C.; Jin, M.; Chang, L.; Dale, B. E.; Balan, V. Alkali-based AFEX pretreatment for the conversion of sugarcane bagasse and cane leaf residues to ethanol. *Biotechnol. Bioeng.* **2010**, *107* (3), 441−450.
(17) Martín, C.; Klinke, H. B.; Thomsen, A. B. Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse. *Enzyme Microb. Technol.* **2007**, *40* (3), 426−432.
(18) Rocha, G. J. d. M; Martin, C.; Soares, I. B.; Souto Maior, A. M.; Baudel, H. M.; Moraes de Abreu, C. A. Dilute mixed-acid pretreatment of sugarcane bagasse for ethanol production. *Biomass Bioenergy* **2011**, *35* (1), 663−670.
(19) Martínez, Á. T.; Ruiz-Dueñas, F. J.; Martínez, M. J.; del Río, J. C.; Gutiérrez, A. Enzymatic delignification of plant cell wall: from nature to mill. *Curr. Opin. Biotechnol.* **2009**, *20*, 348−357.
(20) Ruiz-Dueñas, F. J.; Martínez, Á. T. Microbial degradation of lignin: how a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this. *Microb. Biotechnol.* **2009**, *2* (2), 164−177.
(21) Sharma, P.; Goel, R.; Capalash, N. Bacterial laccases. *World J. Microbiol. Biotechnol.* **2007**, *23* (6), 823−832.
(22) Xu, F. Applications of oxidoreductases: recent progress. *Ind. Biotechnol.* **2005**, *1* (1), 38−50.
(23) Gutiérrez, A.; Rencoret, J.; Cadena, E. M.; Rico, A.; Barth, D.; del Río, J. C.; Martínez, Á. T. Demonstration of laccase-based removal of lignin from wood and non-wood plant feedstocks. *Bioresour. Technol.* **2012**, *119*, 114−122.
(24) Rico, A.; Rencoret, J.; del Rio, J.; Martinez, A.; Gutierrez, A. Pretreatment with laccase and a phenolic mediator degrades lignin and enhances saccharification of Eucalyptus feedstock. *Biotechnol. Biofuels* **2014**, *7* (1), 6.
(25) Rico, A.; Rencoret, J.; del Río, J.; Martínez, A.; Gutiérrez, A. In-depth 2D NMR study of lignin modification during pretreatment of Eucalyptus wood with laccase and mediators. *BioEnergy Res.* **2015**, *8*, 211−230.
(26) Rencoret, J.; Pereira, A.; del Río, J. C.; Martínez, A. T.; Gutiérrez, A. Laccase-mediator pretreatment of wheat straw degrades lignin and improves saccharification. *BioEnergy Res.* **2016**, *9* (3), 917−930.
(27) Euring, M.; Trojanowski, J.; Horstmann, M.; Kharazipour, A. Studies of enzymatic oxidation of TMP-fibers and lignin model compounds by a Laccase−Mediator-System using different ^{14}C and ^{13}C techniques. *Wood Sci. Technol.* **2012**, *46* (4), 699−708.
(28) Camassola, M.; Dillon, A. J. P. Biological pretreatment of sugar cane bagasse for the production of cellulases and xylanases by *Penicillium echinulatum*. *Ind. Crops Prod.* **2009**, *29* (2−3), 642−647.
(29) Machado, A. d. S.; Ferraz, A. Biological pretreatment of sugarcane bagasse with basidiomycetes producing varied patterns of biodegradation. *Bioresour. Technol.* **2017**, *225*, 17−22.
(30) Pandey, A.; Soccol, C. R.; Nigam, P.; Soccol, V. T. Biotechnological potential of agro-industrial residues. I: sugarcane bagasse. *Bioresour. Technol.* **2000**, *74* (1), 69−80.
(31) Lomascolo, A.; Record, E.; Herpoël-Gimbert, I.; Delattre, M.; Robert, J. L.; Georis, J.; Dauvrin, T.; Sigoillot, J. C.; Asther, M. Overproduction of laccase by a monokaryotic strain of *Pycnoporus cinnabarinus* using ethanol as inducer. *J. Appl. Microbiol.* **2003**, *94* (4), 618−624.
(32) Alves, A. M. C. R.; Record, E.; Lomascolo, A.; Scholtmeijer, K.; Asther, M.; Wessels, J. G. H.; Wösten, H. A. B. Highly efficient production of laccase by the basidiomycete *Pycnoporus cinnabarinus*. *Appl. Environ. Microbiol.* **2004**, *70* (11), 6379−6384.
(33) Kapoor, R. K.; Rajan, K.; Carrier, D. J. Applications of *Trametes versicolor* crude culture filtrates in detoxification of biomass pretreatment hydrolyzates. *Bioresour. Technol.* **2015**, *189*, 99−106.
(34) Tappi. *Tappi Test Methods*; Tappi Press: Norcoss, GA, 2004−2005.
(35) Selvendran, R. R.; March, J. F.; Ring, S. G. Determination of aldoses and uronic acid content of vegetable fiber. *Anal. Biochem.* **1979**, *96* (2), 282−92.
(36) Rencoret, J.; Marques, G.; Gutierrez, A.; Nieto, L.; Santos, J. I.; Jimenez-Barbero, J.; Martinez, A. T.; del Rio, J. C. HSQC-NMR analysis of lignin in woody (*Eucalyptus globulus* and *Picea abies*) and non-woody (*Agave sisalana*) ball-milled plant materials at the gel state. *Holzforschung* **2009**, *63*, 691−698.
(37) Kim, H.; Ralph, J.; Akiyama, T. Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d_6. *BioEnergy Res.* **2008**, *1* (1), 56−66.
(38) Kupče, E.; Freeman, R. Compensated adiabatic inversion pulses: Broadband INEPT and HSQC. *J. Magn. Reson.* **2007**, *187*, 258−265.
(39) Lundquist, K.; Parkås, J. Different types of phenolic units in lignins. *Bioresources* **2011**, *6*, 920−926.
(40) Pu, Y.; Cao, S.; Ragauskas, A. J. Application of quantitative ^{31}P NMR in biomass lignin and biofuel precursors characterization. *Energy Environ. Sci.* **2011**, *4* (9), 3154−3166.
(41) del Río, J. C.; Prinsen, P.; Rencoret, J.; Nieto, L.; Jiménez-Barbero, J.; Ralph, J.; Martínez, Á. T.; Gutiérrez, A. Structural characterization of the lignin in the cortex and pith of elephant grass (*Pennisetum purpureum*) stems. *J. Agric. Food Chem.* **2012**, *60* (14), 3619−3634.
(42) Rencoret, J.; Marques, G.; Gutierrez, A.; Ibarra, D.; Li, J.; Gellerstedt, G.; Santos, J. I.; Jimenez-Barbero, J.; Martinez, A. T.; del Río, J. C. Structural characterization of milled wood lignins from different eucalypt species. *Holzforschung* **2008**, *62* (5), 514−526.
(43) Heap, L.; Green, A.; Brown, D.; van Dongen, B.; Turner, N. Role of laccase as an enzymatic pretreatment method to improve lignocellulosic saccharification. *Catal. Sci. Technol.* **2014**, *4* (8), 2251−2259.
(44) Palonen, H.; Viikari, L. Role of oxidative enzymatic treatments on enzymatic hydrolysis of softwood. *Biotechnol. Bioeng.* **2004**, *86*, 550−557.
(45) Cañas, A. I.; Camarero, S. Laccases and their natural mediators: biotechnological tools for sustainable eco-friendly processes. *Biotechnol. Adv.* **2010**, *28* (6), 694−705.
(46) Babot, E. D.; Rico, A.; Rencoret, J.; Kalum, L.; Lund, H.; Romero, J.; del Río, J. C.; Martínez, Á. T.; Gutiérrez, A. Towards industrially-feasible delignification and pitch removal by treating paper pulp with *Myceliophthora thermophila* laccase and a phenolic mediator. *Bioresour. Technol.* **2011**, *102* (12), 6717−6722.

DOI: 10.1021/acssuschemeng.7b01332
ACS Sustainable Chem. Eng. 2017, 5, 7145−7154

(47) Kirk, T.; Farrell, R. Enzymatic "combustion": the microbial degradation of lignin. *Annu. Rev. Microbiol.* **1987**, *41*, 465−505.
(48) Srebotnik, E.; Hammel, K. E. Degradation of nonphenolic lignin by the laccase/1-hydroxybenzotriazole system. *J. Biotechnol.* **2000**, *81* (2−3), 179−188.
(49) Fabbrini, M.; Galli, C.; Gentili, P. Comparing the catalytic efficiency of some mediators of laccase. *J. Mol. Catal. B: Enzym.* **2002**, *16* (5−6), 231−240.
(50) Kawai, S.; Nakagawa, M.; Ohashi, H. Degradation mechanisms of a nonphenolic *β*-*O*-4 lignin model dimer by *Trametes versicolor* laccase in the presence of 1-hydroxybenzotriazole. *Enzyme Microb. Technol.* **2002**, *30*, 482−489.

DOI: 10.1021/acssuschemeng.7b01332
ACS Sustainable Chem. Eng. 2017, 5, 7145−7154

PUBLICACIÓN 3:

Rencoret J., Pereira A., Marques G., del Río J.C., Martínez A.T., Gutiérrez A. (2018) A commercial laccase -mediator system to delignify and improve saccharification of the fast-growing *Paulownia fortunei*. *Holzforschung* (en prensa)

DE GRUYTER Holzforschung
Wood Research & Technology

A commercial laccase-mediator system to delignify and improve saccharification of the fast-growing *Paulownia fortunei*

Journal:	*Holzforschung - International Journal of the Biology, Chemistry, Physics and Technology of Wood*
Manuscript ID	HOLZ.2018.0095.R2
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Rencoret, Jorge; Instituto de Recursos Naturales y Agrobiología de Sevilla, Pereira, Antonio; Instituto de Recursos Naturales y Agrobiología de Sevilla Marques, Gisela; Instituto de Recursos Naturales y Agrobiología de Sevilla del Rio, Jose; Instituto de Recursos Naturales y Agrobiologia de Sevilla, IRNAS-CSIC Martinez, Angel; CIB, CSIC, Molecular Microbiology Gutierrez Suarez, Ana; Agrobiologia de Sevilla, Instituto de Recursos Naturales y
Section/Category:	Biochemistry and Biotechnology
Keywords:	pretreatment, paulownia, lignin, 2D-NMR, laccase, saccharification

Short title: **Enzymatic pretreatment of *P. fortunei***

A commercial laccase-mediator system to delignify and improve saccharification of the fast-growing *Paulownia fortunei*

Jorge Rencoret[1,*], Antonio Pereira[1], Gisela Marques[1], José Carlos del Río[1], Ángel T. Martínez[2] and Ana Gutiérrez[1]

[1]Instituto de Recursos Naturales y Agrobiología de Sevilla, CSIC, E-41012-Seville, Spain
[2]Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, E-28040-Madrid, Spain
* Corresponding author: (Tel: +34 954624711; E-mail: jrencoret@irnase.csic.es)

Abstract: It was demonstrated for the first time that a laccase-based enzymatic pretreatment is able to delignify fast-growing paulownia species. The treatment was performed with a commercial low-redox potential laccase isolated from *Myceliophthora thermophila* and methyl syringate (MeS) as a natural phenolic mediator. Up to 24% lignin removal was attained by the laccase-mediator treatment (L/MeS), followed by alkaline peroxide extraction in a multistage sequence. The reduction in lignin content was accompanied by a significant improvement in the subsequent enzymatic saccharification ending up in 38% glucose and 34% xylose yields. The structural modifications of the lignin were analyzed *in situ* by 2D-NMR spectroscopy. A considerable removal of guaiacyl and syringyl lignin units with respect to the carbohydrate signals was visible as well as the cleavage of β-*O*-4′, β-5′ and β-β′ linkages leading to elevated amounts of Cα-oxidized guaiacyl and syringyl units. The presence of oxidized lignin compounds in the filtrates of the enzymatic treatments ─ such as vanillin, vanillic acid, syringaldehyde and syringic acid ─ conclusively demonstrates the ability of L/MeS treatment to depolymerize the lignin in paulownia wood.

Keywords: pretreatment; paulownia; lignin; 2D-NMR; laccase; saccharification

Introduction

Paulownia genus comprises of nine species and a few hybrids native to China and East Asian (Zhu et al. 1986), which are grown commercially for the production of hardwood timber (Bergmann 1998), while *Paulownia* is a fast-growing tree with a high biomass production (Jiménez et al. 2005). Its properties such a lightweight, strength, insulation, fast drying, high ignition point and rot resistance makes it popular for house construction and furniture making. It was also investigated as a raw material for the production of chemical pulp (Jiménez et al. 2005; Caparrós et al. 2007, 2008). *Paulownia fortunei* is the most suitable species to this purpose (Rai et al. 2000). *P. fortunei* shows extraordinary high growth rates under suitable conditions (Ede et al. 1997), reaching up to 15-20 m high in only 5-7 years, and annual productions as high as 150 t ha^{-1} y^{-1} (Jiménez et al. 2005).

Paulownia also has a potential use as energy crop for the production of bioethanol (Ye et al. 2016; Domínguez et al. 2017) by enzymatic hydrolysis (Chandra et al. 2007) and subsequent fermentation by yeasts (Park et al. 2013) or bacteria (Ng et al. 1981). To this purpose, the cross-linked macro-molecular assembly of the cell wall must be submitted to a pretreatment (Chen 2014). Especially lignins limit the enzymatic hydrolysis by steric hindrance of the enzymes access to the polysaccharides and their inactivation (Kumar and Wyman 2009; Rahikainen et al. 2013).

Paulownia wood contains ~24% lignin, which is composed of guaiacyl (G) and syringyl (S) units with an S/G ratio of 0.66 (Rencoret et al. 2009a). Different physical and chemical pretreatments have been proposed for a better saccharification of paulownia wood, such as dilute acid, alkali, ultrasonic-assisted alkali treatments (Ye et al. 2015, 2016), autohydrolysis (Domínguez et al. 2017), and steam explosion (Radeva et al. 2012). Biological pretreatments are also possible via ligninolytic fungi or their enzymes, but these methods have not yet been investigated on paulownia.

The aim of the present study is to evaluate the pretreatment of paulownia wood by commercially available laccase-mediator (L/M) system to improve the subsequent saccharification. The thermostable laccase from the fungus *M. thermophila* will be applied from Novozymes (Bagsvaerd, Denmark) that has been cloned and expressed in *Aspergillus oryzae* (Xu et al. 1996; Berka et al. 1997). Methyl syringate (MeS) will serve as mediator, which is obtained from syringic acid present in pulp and paper side-streams (Rosado et al. 2012). MeS is cheaper and less toxic than synthetic mediators such as HBT, violuric acid or ABTS. The L/MeS treatment was proved to be highly effective on eucalypt wood (Rico et al. 2014), which contains a syringyl-rich lignin (Rencoret et al. 2008) that is easier to degrade under alkaline conditions (González-Vila et al. 1999; Shimizu et al. 2017). The modification of the structure of the lignin polymer in the pretreated paulownia will be monitored by 2D-NMR spectroscopy of the whole sample at the gel state (Kim et al. 2008; Rencoret et al. 2009b), and the effect of the L/MeS treatment on the saccharification yield will be reported.

Material and methods

Paulownia wood, enzymes and mediator: 3-year-old *P. fortunei* trees were provided by the University of Huelva (Spain). The wood was manually debarked, chipped, air-dried and ground in a knife mill IKA MF1O to pass 1 mm screen, and then finely milled in a planetary mill Retsch PM100 at 400 rpm for 2 h (including pause times to prevent sample heating), using a 500 mL agate jar and agate balls (20 × 20 mm). The commercial (recombinant) fungal laccase from *M. thermophila* (Novozym 51003) was supplied by Novozymes. The enzymatic activity was measured as initial velocity during oxidation of 5 mM ABTS from Roche to its cation radical (ε_{436} 29300 M^{-1} cm^{-1}) in 0.1 M sodium acetate (pH 5) at 24°C. The laccase activity was 1000 U mL^{-1}. One activity unit (U) is defined as the amount of enzyme transforming 1 μmol of ABTS per min. MeS (methyl 4-hydroxy-3,5-dimethoxybenzoate from Alfa Aesar (Karlsruhe, Germany) served as the redox mediator.

L/MeS treatments: A sequence of four L/MeS treatments was applied, each one followed by an alkaline peroxide extraction step. Laccase doses of 50 U g^{-1} were assayed, together with 3% MeS (% is b.o. dry wood). Paulownia samples (4 g) at 6% consistency (w:w) in 50 mM NaH_2PO_4 (pH 6.5) were placed into 200 mL pressurized bioreactors (Labomat, Mathis, Switzerland) and treated under O_2 atmosphere (2 bar), in a thermostatic shaker (adjusted at 50°C and 190 rpm), for 24 h. Then, samples were filtered through a Büchner funnel and washed with 1 L of water. Subsequently, samples at 6% consistency (w:w) were submitted to an alkaline peroxide extraction wit 1% (w:w) NaOH and 3% (w:w) H_2O_2 at 80°C for 90 min in a thermostatic shaker at 140 rpm, followed by water washing. Treatments with laccase alone (50 U g^{-1}), without mediator, and controls without laccase and mediator were also performed. A control treatment with mediator alone was not included in view of the negative results of previous studies. The Klason lignin contents (TAPPI Method T222 om-88, 2004) were corrected for ash.

Saccharification of pretreated samples: After L/MeS treatment, the samples were hydrolyzed in an enzyme cocktail of commercial enzymes (Novozymes) with cellulase (Celluclast 1.5 L; 2 FPU g^{-1}) and β-glucosidase (Novozym 188; 6 U g^{-1}) at 1% solid loading in 3 mL of 100 mM sodium citrate (pH 5) for 72 h at 45°C, in a shaker bath at 140 rpm. The released monosaccharides were determined as alditol acetate derivatives by GC (Selvendran et al. 1979) on an HP 5890 instrument (Hewlett-Packard, Hoofddorp, The Netherlands), as previously described (Rencoret et al. 2017). Chromatographic peaks were quantified by area, and different standards (including glucose and xylose, among others) were used to elaborate calibration curves. Duplicate experiments were performed in terms of of L/MeS treatment (including control, laccase and laccase-MeS) and glucose and xylose release, and Klason lignin contents were analysed in triplicate measurements. At the 95% confidence level, the enzymatic experiments are smaller than the differences found between the control, laccase alone, and L/MeS treatments. Moreover, ANOVA experiments between subjects were

performed. Post hoc pairwise comparisons, using the Tukey HSD test, were also performed. The data from both enzymatic and technical replicates were averaged.

2D-NMR analyses: The 2D HSQC NMR experiments were performed at the gel state, which is an *in situ* analysis of the whole cell-wall (Kim et al. 2008, Rencoret et al. 2009b). This approach does not require a previous lignin isolation and avoids possible alterations and material losses during the isolation process. These experiments provided also structural information on the hemicelluloses in the cell wall. 70 mg of ball-milled samples (and filtrate samples) were transferred into 5-mm NMR tubes, and swelled in 1 mL of DMSO-d_6, forming a gel inside the tube (Kim et al. 2008; Rencoret et al. 2009b). Heteronuclear single quantum coherence (HSQC) 2D-NMR spectra were recorded at 300 K on a Bruker AVANCE III 500 MHz spectrometer equipped with a 5 mm TCI gradient cryoprobe with inverse geometry. An adiabatic HSQC pulse sequence (Bruker standard 'hsqcetgpsisp.2'), which enabled a semiquantitative analysis of the different ^{1}H-^{13}C correlation signals (Kupče and Freeman, 2007), was utilized. HSQC spectra were acquired from 10 to 0 ppm (5000 Hz) in F2 (^{1}H) using 1000 data points for an acquisition time (AQ) of 100 ms, an interscan delay (D1) of 1 s, and from 200 to 0 ppm (25,168) in F1 (^{13}C) using 256 increments of 32 scan, for a total acquisition time of 2 h 34 min. The $^{1}J_{CH}$ used was 145 Hz. Processing used typical matched Gaussian apodization in ^{1}H and a squared cosine bell in ^{13}C. The central solvent peak was used as an internal reference (δ_C/δ_H 39.5/2.49). Lignin and carbohydrate correlation signals in the HSQC spectra were assigned by comparison with the literature (Rencoret et al. 2009a; 2009b; Kim et al. 2014). The ^{1}H-^{13}C correlation signals from the aromatic region of the spectrum were used to estimate the content of lignin (relative to the content of amorphous carbohydrates, estimated from the anomeric xylose and glucose signals), and the lignin composition in terms of G, S and oxidized S (S′) and G (G′) units. The C_α-H_α correlation signals in the aliphatic-oxygenated region were used to estimate the abundance of the various lignin inter-unit linkages whereas the C_γ-H_γ correlation signals were used to estimate the

abundance of the cinnamyl alcohol end-units. Likewise, $S_{2,6}$ (and $S'_{2,6}$) and G_2 (and G'_2) signals were used to estimate the relative abundances of the aromatic units – as signals $S_{2,6}$ and $S'_{2,6}$ involve two proton-carbon pairs, their volume integrals were halved. The content of Cα-oxidized S-lignin units in the HSQC spectrum of paulownia treated with laccase and mediator was corrected for the contribution of MeS to the signal at δ_C/δ_H 106.2/7.20, which was estimated from the integral of its characteristic signal at δ_C/δ_H 52/3.8 ppm.

Analyses of enzymatic filtrates: The filtrates obtained after each enzymatic treatment were combined (from the four cycles), lyophilized and subsequently extracted with chloroform by sonication (20 min). The solution was centrifuged (10000 rpm for 25 min) and the supernatant, which contains low molecular weight lignin-derived compounds, was then collected by decantation. The extraction process was repeated three times, using fresh chloroform each time and the supernatants were combined. The chloroform was removed by rotary evaporation at 40°C and the compounds were then silylated with bis(trimethylsilyl)trifluoroacetamide (BSTFA)-pyridine (2:1 v/v) and analyzed by GC-MS on a Varian Saturn 4000 equipment. The GC column: 15 m × 0.25 mm i.d., 0.1 μm film thickness, DB5-HT (J&W Scientific). The temperature program: 50°C → 90°C (30°C min^{-1}), holding time 2 min, → 250°C (8°C min^{-1}), holding time 2 min. The injector and transfer line temperatures were 250°C and 300°C, respectively. He was the carrier gas (2 mL min^{-1}). The chloroform-insoluble fractions of the filtrates, which contain lignin fragment with higher molecular weights, were analyzed by 2D-NMR under the same conditions as described above.

Result and discussion

Delignification of paulownia wood by the enzymatic pretreatment

The Klason lignin content of the paulownia control, processed as the full enzymatic treatment but without laccase and mediator, was only slightly reduced from 23.8 to 22.0% with respect to the initial paulownia lignin content (Table 1). This is the effect of alkaline peroxide

extractions. The treatment with laccase alone only resulted in a 5% lignin reduction compared to the control sample. This low efficiency of laccase alone is well known (Lai, 1992). The pretreatment with L/MeS, however, resulted in a lignin decrement up to 24%, with respect to the control, but this delignification degree is lower than reported about eucalypt wood treatment under the same experimental conditions (47% lignin removal). This difference may be due to the high S-units content of eucalypt wood lignin compared to the G-units-rich and condensed paulownia lignin.

Saccharification of pretreated paulownia

The percentages of glucose and xylose released by saccharification of treated paulownia samples are listed in Table 1. The saccharification results indicate that there is a direct correlation between the lignin removal and the higher glucose and xylose yields. The pretreatment with laccase alone enhanced the glucose and xylose yields up to 6% and 7%, respectively, but the L/MeS treatment led to glucose and xylose increase up to 38% and 34%, respectively. Rico et al. (2014) found similar glucose (41%) and xylose (37%) yield increments with eucalypt wood after L/MeS under the same experimental conditions.

2D-NMR analysis

The full HSQC NMR spectra of the samples, including those resulting from the treatment with L/MeS, L alone, and the corresponding control are shown in Figure 1. A pronounced lignin removal occurred after L/MeS pretreatment, as evidenced by the lower lignin signals (colored in red) after the treatments, in comparison with the signals from carbohydrates (in cyan color) that remained mostly unchanged (Figure 1c), although some lignin degradation can also be seen in the spectrum of L treated samples (Figure 1b). More detailed information was gained from these HSQC spectra after increasing their intensities and analyzing the aliphatic oxygenated and the aromatic regions individually (Figure 2). The aliphatic

oxygenated region (δ_H/δ_C, 2.5-6.0/50-110) of the spectra shows correlation signals from both lignin (side-chain linkages and aromatic methoxy groups) and carbohydrates, which are mainly from hemicelluloses and amorphous cellulose as crystalline cellulose is "invisible" to the HSQC analysis in the gel state. The lignin and carbohydrate cross-signals assigned in the HSQC spectra are listed in Table 2, and the lignin units and substructures identified are depicted in Figure 3. The carbohydrate signals mainly correspond to xylans, both non-acetylated (X) and acetylated (X′), and 4-*O*-methyl-α-D-glucuronic acid, in agreement with previous work that reported an *O*-acetyl-(4-*O*-methylglucurono)xylan as being the main heteroxylan in paulownia (Gonçalves et al. 2008). The lignin signals in this region of the spectra corresponded to MeO groups and different lignin inter-unit linkages such as β-*O*-4′ alkyl-aryl ethers (A), β-5′ phenylcoumarans (B), and β-β′ resinols (C). The aromatic region (δ_H/δ_C, 6.0-8.0/100-125) of the spectra included signals from G and S lignin units, as well as the corresponding Cα-oxidized lignin units (G′ and S′).

The HSQC spectrum of the sample after the enzymatic treatment with laccase alone shows noticeable differences in the lignin correlation signals, in comparison to the control spectrum, whereas the carbohydrates signals remain mostly unchanged. The considerable diminution of signals from the main lignin inter-units linkages is the most remarkable feature and the relative signal increment from Cα-oxidized lignin units (G′ and S′). The abundance of β-*O*-4′ alkyl-aryl ether linkages per aromatic units decreased from 51 to 39, whereas the abundance of β-β′ resinol linkages decreased from 4 to 2 per aromatic units and the β-5′ phenylcoumaran linkages were not even detectable in the HSQC spectrum of the sample treated with laccase alone (Table 3), where the integrals of HSQC signals are semiquantitatively compared. Likewise, the increase of the signals for Cα-oxidized S-lignin units (S′), together with the appearance of signals corresponding to Cα-oxidized G-lignin units (G′), indicates that *M. thermophila* laccase itself was able to oxidize the phenolic substructures to a certain extent. Again, one should emphasize the G-unit-rich character of

paulownia lignin (Rencoret et al. 2009a). The G-units have more free OH_{phen} groups than the S-units, which are mainly etherified (Lundquist and Parkås 2011; Pu et al. 2011). This could explain the degradation/modification of the lignin polymer observed during the treatment with laccase alone. However, the lignin content decreased only by 5% after L treatment alone.

Interestingly, the HSQC spectrum of the sample treated via L/MeS (Figure 2c,f) revealed a high degree of lignin degradation. The intensity of most of the lignin signals strongly decreased, while the signals from carbohydrates remained mostly unchanged (Figure 2c). Even though, signals from acetylated xylans (X'_2 and X'_3) increased after L/MeS pretreatment, as occurred with other lignocellulosic materials, such as eucalypt wood (Gutiérrez et al. 2012) and sugarcane byproducts (Rencoret et al. 2017), probably due to the better mobility of these groups after lignin removal. This is reflected by the semiquantitative NMR analysis (Table 3) that showed a relative increase of sugar units, with respect to lignin aromatic units, from 58.3% (control sample) to 74.1% (L/MeS pretreatment). The signals of β-*O*-4′ alkyl-aryl ether lignin substructures (A), which were the most intense in the HSQC of the control sample, were hardly visible after the enzymatic pretreatment, whereas the signals from β-5′ phenylcoumarans (B) and β-β′ resinols (C) were not detected at all. These observations confirm the depolymerization of the paulownia lignin. Interestingly, a new correlation signal (Aox_β at 83.0/5.20) corresponding to C_β-H_β correlations in Cα-oxidized β-*O*-4′ alkyl-aryl ether linkages appeared as a result of L/MeS treatment. The formation of this uncleaved ketone is consistent with the changes observed in the aromatic units, described below, and the Cα-oxidation mechanism proposed for the degradation of lignin with the laccase-mediator system (Bourbonnais and Paice, 1990; Li et al. 1999). The aromatic region of the spectrum shows signal decrement from G- and S-units (Figure 2f). A significant part of the lignin are Cα-oxidized, as evidenced from the relative increase in the $S'_{2,6}$ signals (the contribution of $MeS_{2,6}$ correlations was subtracted) and the appearance of G'_2, G'_5 and G'_6 signals. It is obvious that paulownia lignin is degraded by the L/MeS system following the

same mechanism reported for the L/HBT system with non-phenolic lignin model dimers, which occurs via abstraction of a hydrogen atom from the Cα position, forming a ketone group, and ending up the Cα-Cβ breakdown at a later stage (Fabbrini et al. 2002; Kawai et al. 2002). Clearly, the lignin depolymerization mechanism by L/MeS has an oxidative character.

Analysis of pretreatment filtrates by GC-MS and 2D-NMR

The chloroform-soluble fractions of the filtrates were analyzed by GC-MS to determine the presence of low-molecular weight lignin-derived compounds (Figure 4). The presence of such compounds was negligible in the filtrates of control wood, whereas they were clearly observed in the filtrates after L treatment alone and, especially, after L/MeS treatment. The main substances are vanillin, syringaldehyde, vanillic acid and syringic acid. Again, clearly evidences of the oxidative nature of laccase degradation.

The chloroform-insoluble fractions of the filtrates were analyzed by 2D-NMR aiming at the characterisation of oligo- and polymers, which are water insoluble at pH 6.5. There are significant differences among these fractions (Figure 5). While the oligomeric lignin in the control filtrate is less oxidized (higher S/S′ ratio) and preserved the main original inter-unit linkages, the oligomeric lignins in the filtrates after L and L/MeS treatments are more oxidized in the low DP fraction. All these data confirm the extensive oxidative depolymerization of paulownia wood during the L/MeS treatment.

Conclusions

The lignin in paulownia wood was modified and partially removed by pretreatment with recombinant laccase of *M. thermophila* in the presence of MeS as phenolic mediator, in spite of its high G-unit content. The L/MeS system acts selectively on the lignin polymer, leaving the carbohydrate signals in the HSQC spectra practically unaffected. The alterations produced in the lignin moiety further facilitated the access of the hydrolytic enzymes to cell-wall

carbohydrates in the subsequent saccharification step, resulting in higher glucose and xylose yields. The utilization of commercially available L/MeS system has a high application potential as pretreatment for the saccharification of paulownia wood.

Acknowledgements: This study has been funded by the Spanish projects CTQ2014-60764-JIN, AGL2014-53730-R and AGL2017-83036-R (financed by Agencia Estatal de Investigación, AEI, and Fondo Europeo de Desarrollo Regional, FEDER), and the CSIC project 2014-40E-097. We thank Dr. Manuel Angulo (CITIUS, University of Seville) for technical assistance with the NMR experiments.

References

Bergmann, B.A. (1998) Propagation method influences first year field survival and growth of Paulownia. New Forests. 16:251–264.

Berka, R.M., Schneider, P., Golightly, E.J., Brown, S.H., Madden, M., Brown, K.M., Halkier, T., Mondorf, K., Xu, F. (1997) Characterization of the gene encoding an extracellular laccase of *Myceliophthora thermophila* and analysis of the recombinant enzyme expressed in *Aspergillus oryzae*. Appl. Environ. Microbiol. 1997, 63:3151–3157.

Bourbonnais, R., Paice, M. (1990) Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. FEBS Lett. 267:99–102.

Caparrós, S., Ariza, J., Garrote, G., López, F., Díaz, M.J. (2007) Optimization of *Paulownia fortunei* L. autohydrolysis-organosolv pulping as a source of xylooligomers and cellulose pulp. Ind. Eng. Chem. Res. 46:623–631.

Caparrós, S., Díaz, M.J., Ariza, J., López, F., Jiménez, L. (2008) New perspectives for *Paulownia fortunei* L. valorisation of the autohydrolysis and pulping processes. Bioresour. Technol. 99:741–749.

Chandra, M.S., Viswanath, B., Reddy, B.R. (2007) Cellulolytic enzymes on lignocellulosic substrates in solid state fermentation by *Aspergillus niger*. Indian J. Microbiol. 47:323–328.

Chen, H. (2014) Chemical composition and structure of natural lignocellulose. In: Biotechnology of lignocellulose: Theory and practice. Springer Netherlands, Dordrecht. pp. 25–71.

Domínguez, E., Romaní, A., Domingues, L., Garrote, G. (2017) Evaluation of strategies for second generation bioethanol production from fast growing biomass Paulownia within a biorefinery scheme. Appl. Energy 187:777–789.

Ede, F.J., Auger, M., Green, T.G.A. (1997) Optimizing root cutting success in *Paulownia* spp. J. Hortic. Sci. 72:179–185.

Fabbrini, M., Galli, C., Gentili, P. (2002) Comparing the catalytic efficiency of some mediators of laccase. J. Mol. Catal. B Enzym. 16:231–240.

Gonçalves, V.M.F., Evtuguin, D.V., Domingues, M.R.M. (2008) Structural characterization of the acetylated heteroxylan from the natural hybrid *Paulownia elongata*/*Paulownia fortunei*. Carbohydr. Res. 343:256–266.

González-Vila, F.J., Almendros, G., del Río, J.C., Martín, F., Gutiérrez, A., Romero, J. (1999) Ease of delignification assessment of different *Eucalyptus* wood species by pyrolysis (TMAH)-GC/MS and CP/MAS ^{13}C-NMR spectrometry. J. Anal. Appl. Pyrolysis 49:295–305.

Gutiérrez, A., Rencoret, J., Cadena, E.M., Rico, A., Barth, D., del Río, J.C., Martínez, Á.T. (2012) Demonstration of laccase-based removal of lignin from wood and non-wood plant feedstocks. Bioresour. Technol. 119:114–122.

Jiménez, L., Rodríguez, A., Ferrer, J.L., Ṕerez, A., Angulo, V. (2005) Paulownia, a fast-growing plant, as a raw material for paper manufacturing. Afinidad. 62:100–105.

Kawai, S., Nakagawa, M., Ohashi, H. (2002) Degradation mechanisms of a nonphenolic beta-O-4 lignin model dimer by *Trametes versicolor* laccase in the presence of 1-hydroxybenzotriazole. Enzyme Microb. Technol. 30:482–489.

Kim, H., Ralph, J. (2014) A gel-state 2D-NMR method for plant cell wall profiling and analysis: a model study with the amorphous cellulose and xylan from ball-milled cotton linters. RSC Adv. 4:7549–7560.

Kim, H., Ralph, J., Akiyama, T. (2008) Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d_6. Bioenerg. Res. 1:56–66.

Kumar, R., Wyman, C.E. (2009) Does change in accessibility with conversion depend on both the substrate and pretreatment technology? Bioresour. Technol. 100:4193–4202.

Kupče, E., Freeman, R. (2007) Compensated adiabatic inversion pulses: Broadband INEPT and HSQC. J. Magn. Reson. 187:258–265.

Lai, Y.Z. (1992) Determination of Phenolic Hydroxyl Groups. In: Methods in Lignin Chemistry. Lin, S.Y., Dence, C.W. Springer Berlin Heidelberg, Berlin, Heidelberg. pp. 423–434.

Li, K., Xu, F., Eriksson, K.E. (1999) Comparison of fungal laccases and redox mediators in oxidation of a nonphenolic lignin model compound. Appl. Environ. Microbiol. 65:2654–2660.

Lundquist, K., Parkås, J. (2011) Different types of phenolic units in lignins. BioResources 6, 920–926.

Ng, T.K., Ben-Bassat, A., Zeikus, J.G. (1981) Ethanol Production by Thermophilic Bacteria: Fermentation of Cellulosic Substrates by Cocultures of *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum.* Appl. Environ. Microbiol. 41:1337–1343.

Park, J.M., Oh, B.-R., Seo, J.-W., Hong, W.-K., Yu, A., Sohn, J.-H., Kim, C.H. (2013) Efficient Production of Ethanol from Empty Palm Fruit Bunch Fibers by Fed-Batch Simultaneous Saccharification and Fermentation Using *Saccharomyces cerevisiae.* Appl. Biochem. Biotechnol. 170:1807–1814.

Pu, Y., Cao, S., Ragauskas, A.J. (2011) Application of quantitative ^{31}P NMR in biomass lignin and biofuel precursors characterization. Energy Environ. Sci. 4:3154–3166.

Radeva, G., Valchev, I., Petrin, S., Valcheva, E., Tsekova, P. (2012) Kinetic study of the enzyme conversion of steam exploded *Paulownia tomentosa* to glucose. BioResources 7:412–421.

Rahikainen, J.L., Martin-Sampedro, R., Heikkinen, H., Rovio, S., Marjamaa, K., Tamminen, T., Rojas, O.J., Kruus, K. (2013) Inhibitory effect of lignin during cellulose bioconversion: The effect of lignin chemistry on non-productive enzyme adsorption. Bioresour. Technol. 133:270-278.

Rai, A.K., Singh, S.P., Luxmi, C., Savita, G., 2000. *Paulownia fortunei* - A new fiber source for pulp and paper. Indian Pulp Pap. Tech. Assoc. 12, 51–56.

Rencoret, J., Marques, G., Gutiérrez, A., Ibarra, D., Li, J., Gellerstedt, G., Santos, J.I., Jiménez-Barbero, J., Martínez, Á.T., del Río, J.C. (2008) Structural characterization of milled wood lignins from different eucalypt species. Holzforschung 62:514–526.

Rencoret, J., Marques, G., Gutierrez, A., Nieto, L., Jimenez-Barbero, J., Martinez, A.T., del Río, J.C. (2009a) Isolation and structural characterization of the milled-wood lignin from *Paulownia fortunei* wood. Ind. Crops Prod. 30:137–143.

Rencoret, J., Marques, G., Gutierrez, A., Nieto, L., Santos, J.I., Jimenez-Barbero, J., Martinez, A.T., del Rio, J.C. (2009b) HSQC-NMR analysis of lignin in woody (*Eucalyptus globulus* and *Picea abies*) and non-woody (*Agave sisalana*) ball-milled plant materials at the gel state. Holzforschung 63:691–698.

Rencoret, J., Pereira, A., del Río, J.C., Martínez, Á.T., Gutiérrez, A. (2017) Delignification and saccharification enhancement of sugarcane byproducts by a laccase-based pretreatment. ACS Sustain. Chem. Eng. 5:7145–7154.

Rico, A., Rencoret, J., del Rio, J., Martinez, A., Gutierrez, A. (2014) Pretreatment with laccase and a phenolic mediator degrades lignin and enhances saccharification of Eucalyptus feedstock. Biotechnol. Biofuels 7:6.

Rosado, T., Bernardo, P., Koci K., Coelho, A.V., Robalo, M.P., Martins, L.O. (2012) Methyl syringate: An efficient phenolic mediator for bacterial and fungal laccases. Bioresour. Technol. 124:371–378.

Selvendran, R.R., March, J.F., Ring, S.G. (1979) Determination of aldoses and uronic acid content of vegetable fiber. Anal. Biochem. 96:282–292.

Shimizu, S., Akiyama, T., Yokoyama, T., Matsumoto, Y. (2017) Chemical factors underlying the more rapid β-O-4 bond cleavage of syringyl than guaiacyl lignin under alkaline delignification conditions. J. Wood Chem. Technol. 37: 451-466.

Tappi. Tappi Test Methods 2004−2005. Tappi Press, Norcoss, GA 30092 USA, 2004.

Xu, F., Shin, W.S., Brown ,S.H., Wahleithner, J.A., Sundaram, U.M., Solomon, E.I. (1996) A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability. BBA Protein Struct. Mol. Enzym. 1292:303–311.

Ye, X., Chen, Y. (2015) Kinetics study of enzymatic hydrolysis of Paulownia by dilute acid, alkali, and ultrasonic-assisted alkali pretreatments. Biotechnol. Bioprocess Eng. 20:242–248.

Ye, X., Zhang, Z., Chen, Y., Cheng, J., Tang, Z., Hu, Y. (2016) Physico-chemical pretreatment technologies of bioconversion efficiency of *Paulownia tomentosa* (Thunb.) Steud. Ind. Crops Prod. 87:280–286.

Zhu, Z.H., Chao, C.J., Lu, X.Y., Xiong, Y.G. Paulownia in China: cultivation and utilization. International Development Research Centre, Ottawa, 1986.

FIGURE LEGENDS

Figure 1. 2D-HSQC NMR spectra of paulownia after laccase-mediator treatments followed by alkaline peroxide extractions (4 cycles): Control without enzyme (**a**), treated with 50 U g^{-1} laccase (**b**) and treated with 50 U g^{-1} laccase and 3% MeS (**c**). Correlation signals in reddish-brown color largely correspond to lignin, whereas signals in cyan color belong to carbohydrates.

Figure 2. Expanded aliphatic oxygenated (δ_C/δ_H 50-110/2.5-6.0, top) and aromatic (δ_C/δ_H 100-125/6.0-8.0, bottom) regions of the 2D-HSQC NMR spectra of paulownia samples; Control without enzyme (**a**, **d**), treated with 50 U g^{-1} enzyme (**b, e**) and treated with 50 U g^{-1} enzyme and 3% MeS. The lignin and carbohydrate signal assignments are listed in Table 2 and the lignin structures identified are depicted in Figure 3. Correlation signals of MeS in the HSQC spectrum from laccase-mediator treated paulownia are observed at δ_C/δ_H 52.1/3.82 (C/H in MeS) and δ_C/δ_H 106.4/7.20 (C_2/H_2 and C_6/H_6 in the aromatic ring).

Figure 3. Main lignin units and substructures identified in the 2D-HSQC spectra of treated paulownia samples. **A**) β–*O*–4′ alkyl-aryl ether structures; **Aox**) β–*O*–4′ structures with Cα-oxidized; **B**) β–5′ phenylcoumaran structures; **C**) β–β′ resinol structures; **I**) cinnamyl alcohol end-group; **G**) guaiacyl unit; **S**) syringyl unit; **G′**) Cα-oxidized G unit; **S′**) Cα-oxidized S unit; **MeS**) methyl syringate.

Figure 4. Total ion chromatograms of the chloroform-soluble fraction of filtrates obtained after the enzymatic pretreatment of paulownia with laccase–mediator (**C**), laccase alone (**B**) and the corresponding control (**A**). The lignin-derived simple compounds identified are depicted (a-e). MeS refers to methyl syringate whereas the peak with asterisk mark (*) refers to contamination peak from methyl syringate.

Figure 5. HSQC NMR spectra of lignin compounds in the chloroform-insoluble fraction of filtrates from enzymatic treatment of paulownia with laccase–mediator (**c**), laccase alone (**b**) and the corresponding control (**a**). See Figure 3 for the main lignin-derived structures identified.

Table 1. Laccase-mediator treatment of paulownia wood (material recovery and final lignin content) and subsequent Celluclast hydrolysis (amount of glucose and xylose per 100 g of sample)[*]

	1) Laccase-mediator treatment		2) Celluclast hydrolysis (2 FPU)	
Samples	Recovery (%)	Lignin (%)	Glucose (%)	Xylose (%)
Initial paulownia	100 (4 g)	23.8 ± 0.2	31.5 ± 0.3	6.7 ± 0.1
Control[*]	83 (3.32 g)	22.0 ± 0.1[a]	37.3 ± 0.2[a]	7.3 ± 0.1[a]
Laccase (50 $U \cdot g^{-1}$)[†]	80 (3.21 g)	20.9 ± 0.3[a]	39.4 ± 0.4[b]	7.8 ± 0.1[a]
Laccase (50 $U \cdot g^{-1}$)-MeS (3%)[†]	76 (3.03 g)	16.7 ± 0.2[b]	51.3 ± 0.4[c]	9.8 ± 0.3[b]

[*]Means ± StD presented were obtained from triplicate determination. Letters next to the StD, from the Tukey test, show: no significant differences to the control (a), significant differences (b) and significantly different from both the control and the L-alone results (c), at the 0.05 level. [*]followed by alkaline-peroxide extraction (4 cycles).

Table 2. Assignments of the $^{1}H/^{13}C$ correlation signals in the HSQC spectra of the whole cell-walls from treated paulownia.

Label	δ_C/δ_H (ppm)	Assignment
Lignin signals		
B_β	53.1/3.43	C_β/H_β in phenylcoumaran substructures (**B**)
C_β	53.4/3.04	C_β/H_β in resinols substructures (**C**)
MeO	55.5/3.72	C/H in methoxyls
A_γ	59.6 /3.37 and 3.71	C_γ/H_γ in β–*O*–4′ substructures (**A**)
I_γ	61.3/4.09	C_γ/H_γ in cinnamyl alcohol end-groups (**I**)
B_γ	62.6/3.69	C_γ/H_γ in phenylcoumaran substructures (**B**)
$A_{\alpha(G)}$	70.8/4.73	C_α/H_α in β–*O*–4′ substructures (**A**) linked to a G-unit
C_γ	70.9/4.17 and 3.81	C_γ/H_γ in resinols substructures (**C**)
$A_{\alpha(S)}$	71.8/4.83	C_α/H_α in β–*O*–4′ substructures (**A**) linked to a S-unit
Aox_β	83.0/5.20	C_β/H_β in α-oxidized β–*O*–4′ substructures (**Aox**)
$A_{\beta(G)}$	83.6/4.26	C_β/H_β in β–*O*–4′ substructures linked (**A**) to a G unit
C_α	84.7/4.63	C_α/H_α in resinols substructures (**C**)
$A_{\beta(S)}$	85.8/4.08	C_β/H_β in β–*O*–4′ substructures linked (**A**) to a S unit
B_α	86.7/5.42	C_α/H_α in phenylcoumaran substructures (**B**)
$S_{2,6}$	103.8/6.65	C_2/H_2 and C_6/H_6 in etherified syringyl units (**S**)
$S'_{2,6}$	106.2/7.20	C_2/H_2 and C_6/H_6 in α-oxidized syringyl units (**S′**)
G_2	110.9/6.93	C_2/H_2 in guaiacyl units (**G**)
G'_2	111.7/7.42	C_2/H_2 in α-oxidized guaiacyl units (**G′**)
G_5/G_6	114.9/6.67 and 6.85	C_5/H_5 and C_6/H_6 in guaiacyl units (**G**)
	118.8/6.76	
G'_5	115.0/6.94	C_5/H_5 in α-oxidized guaiacyl units (**G′**)
G'_6	122.7/7.54	C_6/H_6 in α-oxidized guaiacyl units (**G′**)
Carbohydrate signals		
X_5	63.0/3.16 and 3.87	C_5/H_5 in xylopyranose units
X_2	72.5/3.03	C_2/H_2 in xylopyranose units
X'_2	73.0/4.46	C_2/H_2 in 2-*O*-acetylated xylopyranose units
X_3	73.9/3.24	C_3/H_3 in xylopyranose units
X'_3	74.7/4.79	C_3/H_3 in 3-*O*-acetylated xylopyranose units
X_4	75.3/3.49	C_4/H_4 in xylopyranose units
U_4	81.0/3.07	C_4/H_4 in 4-*O*-methyl-α-D-glucuronic acid
$\alpha X_{1(R)}$	91.9/4.88	C_1/H_1 in α-D-xylopyranoside (R) [α-D-glucopyranoside (R)]
$\beta X_{1(R)}$	96.5/4.31	C_1/H_1 in β-D-xylopyranoside (R) [β-D-glucopyranoside (R)]
U_1	97.1/5.15	C_1/H_1 in 4-*O*-methyl-α-D-glucuronic acid
X'_1	99.4/4.48	C_1/H_1 in 2-*O*-acetylated xylopyranose units
X_1	101.5/4.26	C_1/H_1 in xylopyranose units
Gl_1	102.9/4.16	C_1/H_1 in glucopyranose units

Table 3. Semiquantitative NMR analysis of paulownia treated with *M. thermophila* laccase (50 $U \cdot g^{-1}$) and MeS (3%), and laccase alone, compared with control without enzyme and mediator, including sample composition and linkages/end-groups, from HSQC spectra in Figure 2.

Sample composition[*]	**Control**	**Laccase**	**L/MeS**
Syringyl lignin units (**S**)	15.6 (37)	13.9 (42)	9.3 (36)
C_α-oxidized S units (**S′**)	2.6 (6)	5.7 (17)	5.7 (22) [§]
Guaiacyl lignin units (**G**)	23.5 (56)	11.6 (35)	6.2 (24)
C_α-oxidized G units (**G′**)	0 (0)	2.0 (6)	4.7 (18)
Total lignin	*41.7 (100)*	*33.2 (100)*	*25.9 (100)*
Sugar units	58.3	66.8	74.1
Total	100	100	100
Lignin S/G ratio	0.8	1.4	1.4
Linkages and end groups[#]:			
β-*O*-4′ ethers (**A**)	51 (81)	39 (95)	22 (69)
β-*O*-4′ ethers (**A**) Cα oxidized	0 (0)	0 (0)	9 (30)
Phenylcoumarans (B)	7 (11)	0 (0)	0 (0)
Resinols (**C**)	4 (6)	2 (5)	1 (4)
Cinnamyl end-groups (**I**)	1 (2)	0 (0)	0 (0)
Total	64 (100)	41 (100)	31 (100)

[*]Sample composition represents the molar amount of normal (H, G, and S) and Cα-oxidized (G′ and S′) lignin units, and sugar units (mainly xylose and glucose) from the integration of anomeric carbon signals (relative percentages of lignin units are shown in parentheses). [§]The contribution of MeS was subsracted. [#]The percentages of lignin linkages involved in substructures A, B and C, and cinnamyl alcohol end-groups (I) are referred to the total aromatic (G + G′+ S + S′) lignin units (% relative to 100 linkages/end-groups are provided in parentheses) obtained from integration of aliphatic oxygenated signals.

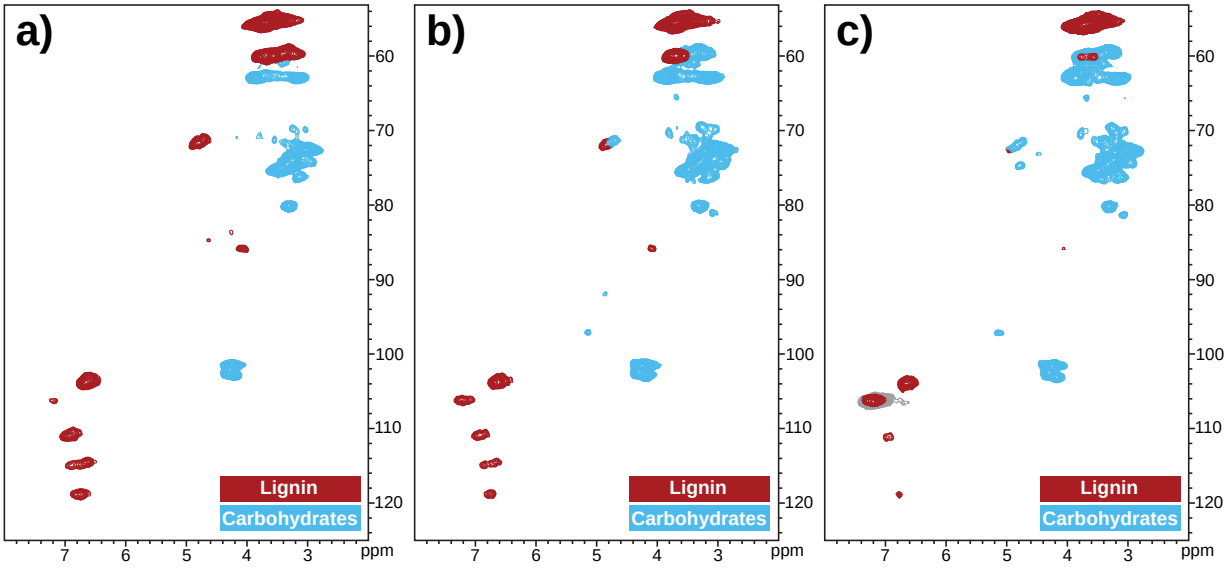


Figure 1

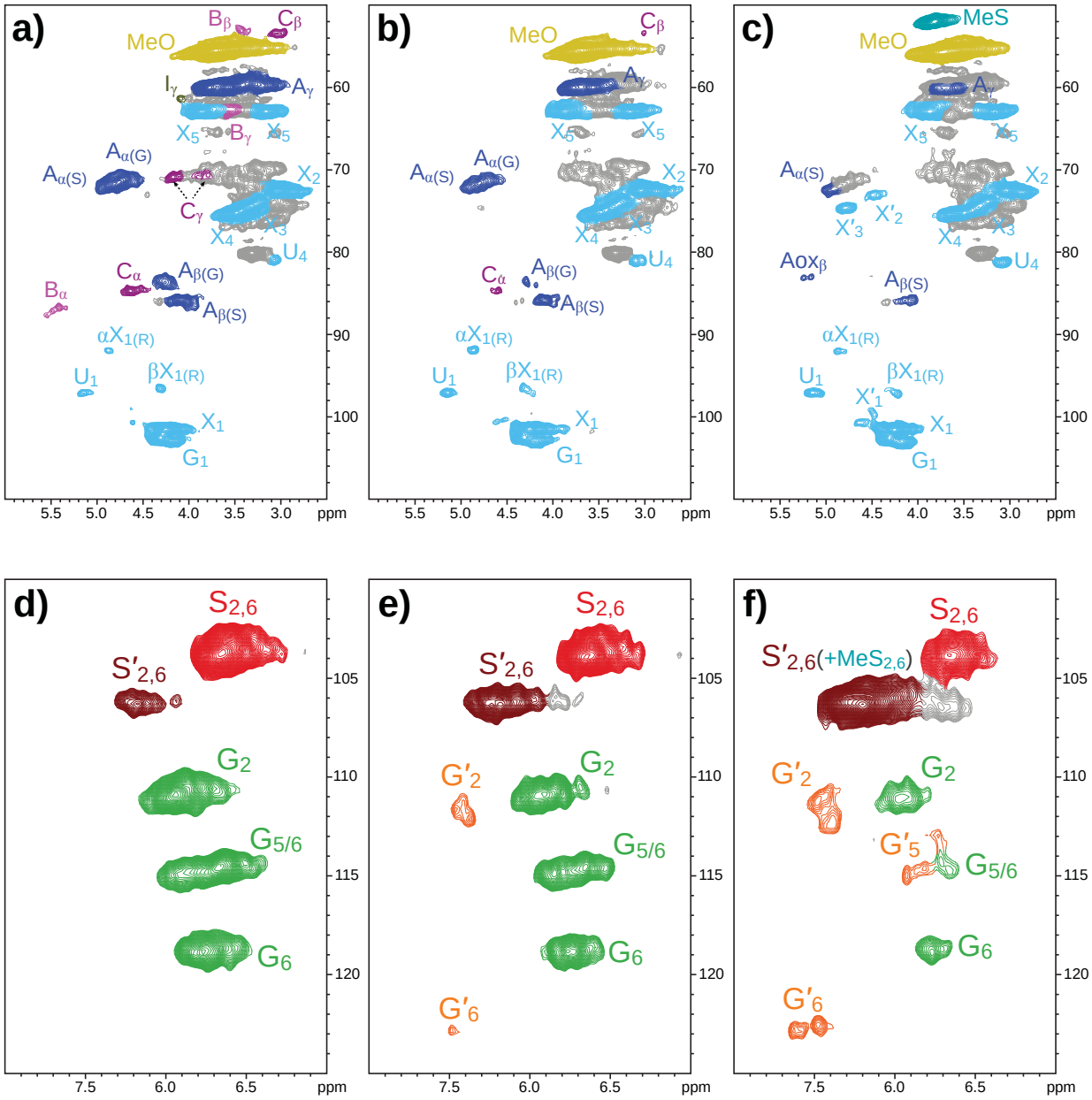

a)
b)
c)
d)
e)
f)
MeO
MeS
Bβ
Cβ
Iγ
Aγ
X5
Bγ
Aα(G)
Aα(S)
X2
Cγ
X4
X3
U4
Cα
Aβ(G)
Aβ(S)
Bα
αX1(R)
βX1(R)
U1
X1
G1
Aoxβ
X'2
X'3
X'1
S2,6
S'2,6
S'2,6(+MeS2,6)
G2
G'2
G5/6
G'5
G6
G'6
ppm

Figure 2

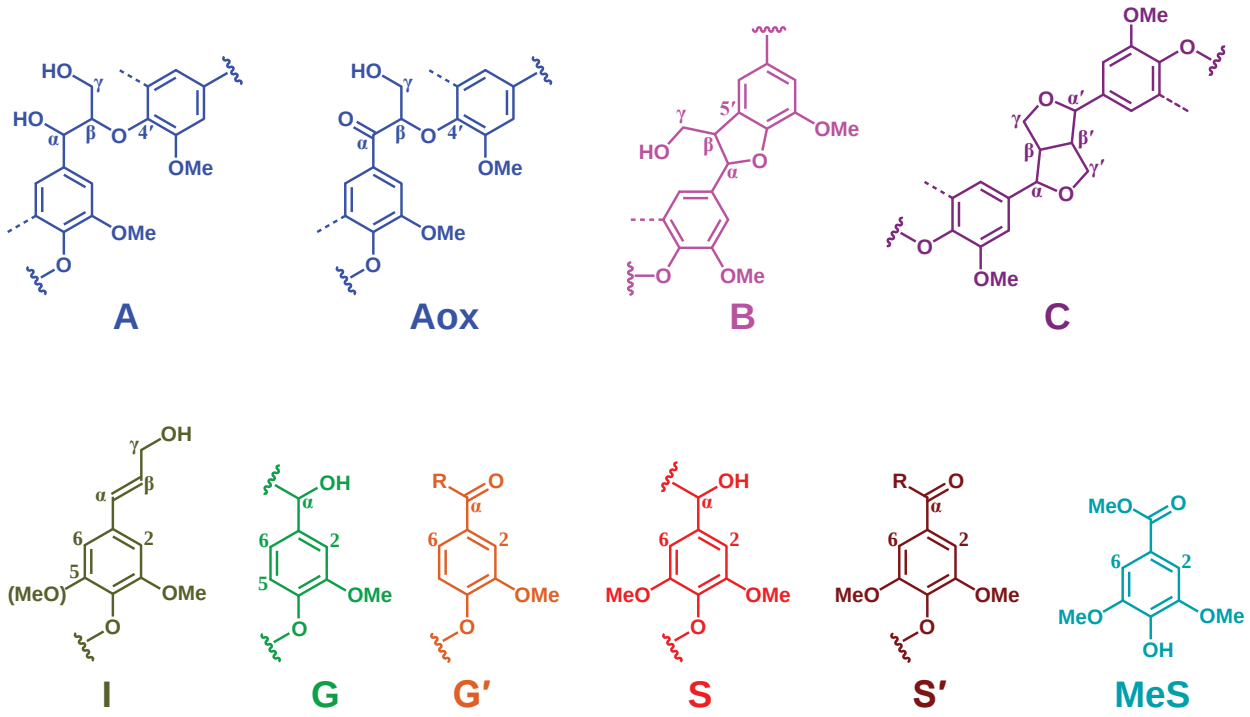


Figure 3

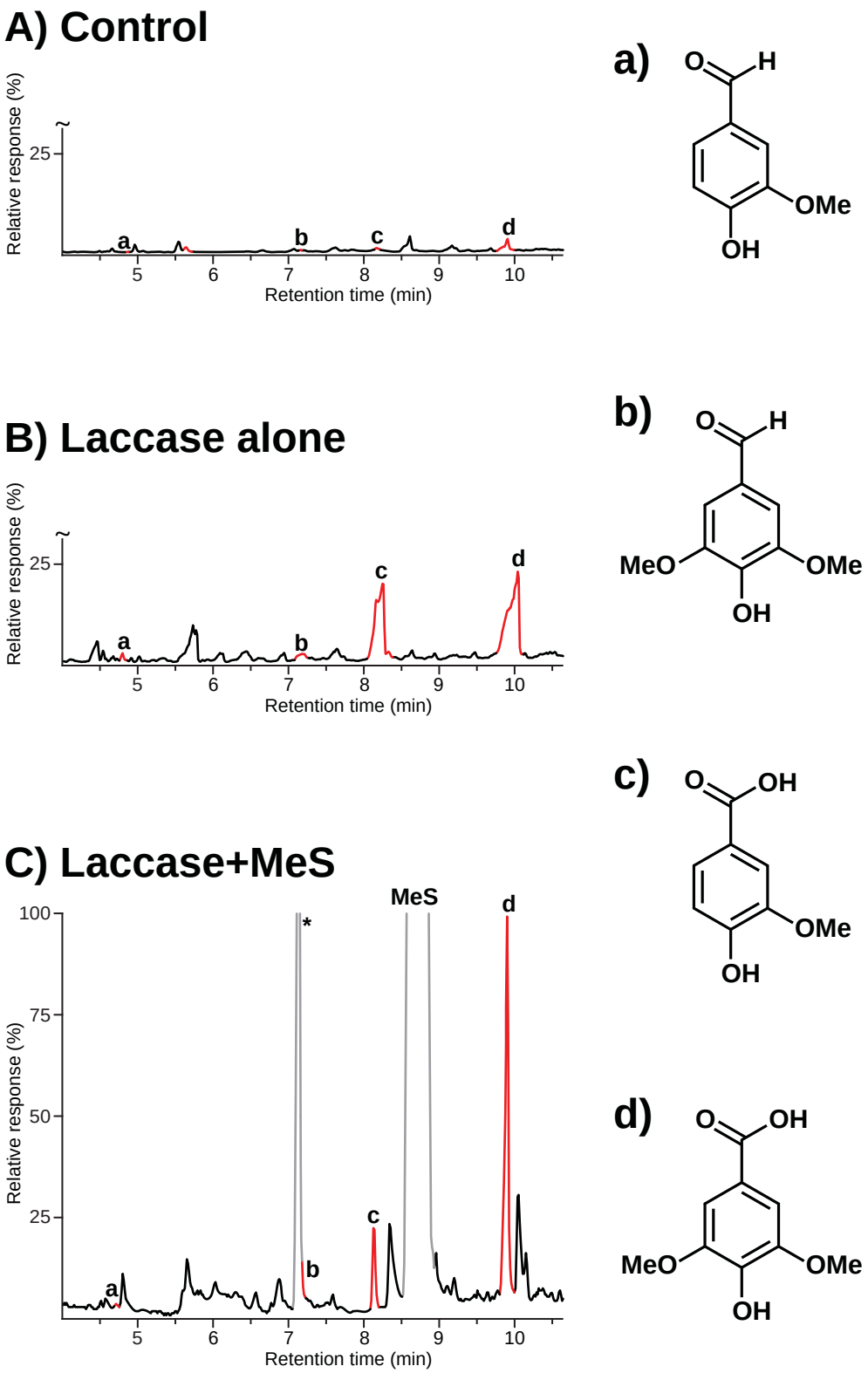

A) Control
Relative response (%)
25
a
b
c
d
5
6
7
8
9
10
Retention time (min)
a)
O
H
OMe
OH
B) Laccase alone
Relative response (%)
25
a
b
c
d
5
6
7
8
9
10
Retention time (min)
b)
O
H
MeO
OMe
OH
c)
O
OH
OMe
OH
C) Laccase+MeS
100
75
50
25
Relative response (%)
*
MeS
a
b
c
d
5
6
7
8
9
10
Retention time (min)
d)
O
OH
MeO
OMe
OH

Figure 4

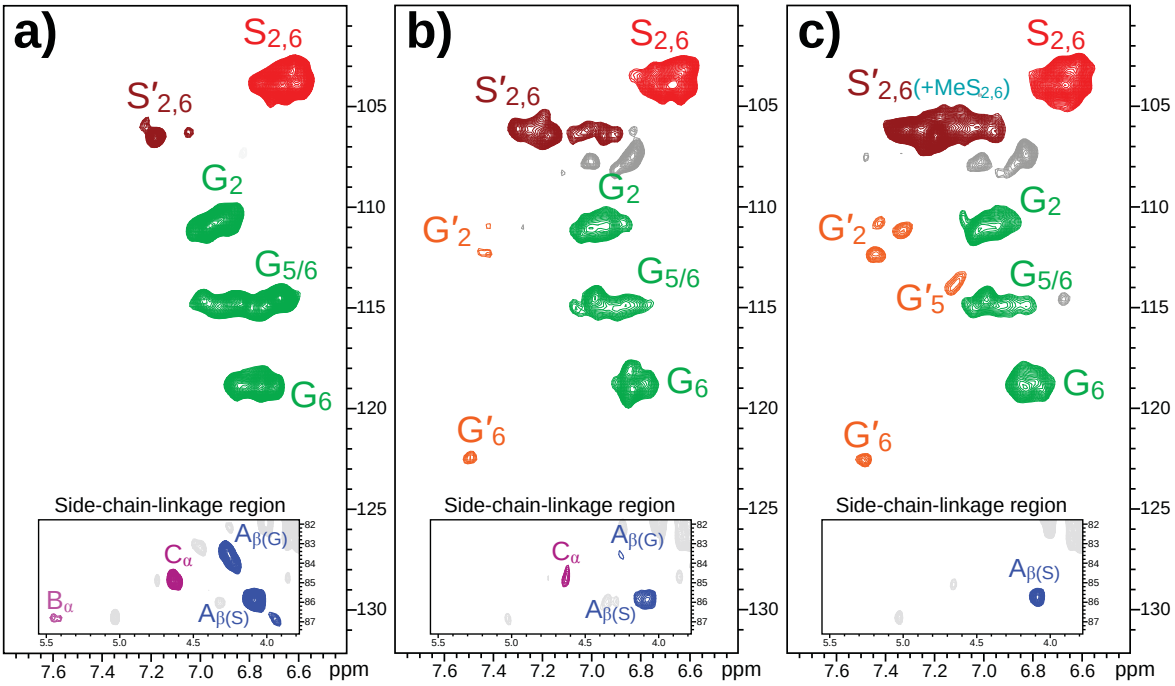

a)
S2,6
S′2,6
G2
G5/6
G6
Side-chain-linkage region
Aβ(G)
Cα
Bα
Aβ(S)
b)
S2,6
S′2,6
G2
G′2
G5/6
G6
G′6
Side-chain-linkage region
Aβ(G)
Cα
Aβ(S)
c)
S2,6
S′2,6(+MeS2,6)
G2
G′2
G′5
G5/6
G6
G′6
Side-chain-linkage region
Aβ(S)
105
110
115
120
125
130
7.6
7.4
7.2
7.0
6.8
6.6
ppm

Figure 5

PUBLICACIÓN 4:

Pereira A., Hoeger I.C., Ferrer A., Rencoret J., del Río J.C., Kruus K., Rahikainen J., Kellock M., Gutierrez A., Rojas O.J. (2017) Lignin films from spruce, eucalyptus, and wheat straw studied with electroacoustic and optical sensors: effect of composition and electrostatic screening on enzyme binding. *Biomacromolecules* 18: 1322-1332.

Article

pubs.acs.org/Biomac

Lignin Films from Spruce, Eucalyptus, and Wheat Straw Studied with Electroacoustic and Optical Sensors: Effect of Composition and Electrostatic Screening on Enzyme Binding

Antonio Pereira,[†,‡] Ingrid C. Hoeger,[‡] Ana Ferrer,[‡] Jorge Rencoret,[†] José C. del Rio,[†] Kristiina Kruus,[§] Jenni Rahikainen,[§] Miriam Kellock,[§] Ana Gutiérrez,[†] and Orlando J. Rojas*[,‡,⊥]

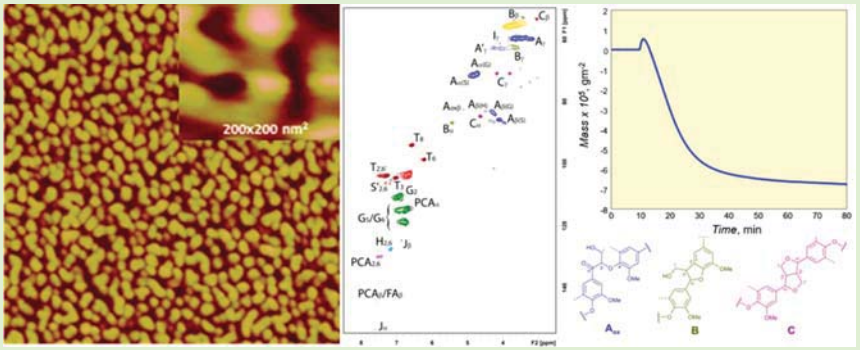
[†]Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS), CSIC, Avenida de la Reina Mercedes, 10, E-41012 Sevilla, Spain

[‡]Departments of Forest Biomaterials and Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, North Carolina 27695, United States

[§]VTT Technical Research Centre of Finland Ltd, P.O. Box 1000, FI-02044 Espoo, Finland

[⊥]Department of Bioproducts and Biosystems, School of Chemical Engineering, Aalto University, FI-00076 Espoo, Finland

Supporting Information



ABSTRACT: Lignins were isolated from spruce, wheat straw, and eucalyptus by using the milled wood lignin (MWL) method. Functional groups and compositional analyses were assessed via 2D NMR and ^{31}P NMR to realize their effect on enzyme binding. Films of the lignins were fabricated and ellipsometry, atomic force microscopy, and water contact angle measurements were used for their characterization and to reveal the changes upon enzyme adsorption. Moreover, lignin thin films were deposited on quartz crystal microgravimetry (QCM) and surface plasmon (SPR) resonance sensors and used to gain further insights into the lignin−cellulase interactions. For this purpose, a commercial multicomponent enzyme system and a monocomponent *Trichoderma reesei* exoglucanase (CBH-I) were considered. Strong enzyme adsorption was observed on the various lignins but compared to the multicomponent cellulases, CBH-I displayed lower surface affinity and higher binding reversibility. This resolved prevalent questions related to the affinity of this enzyme with lignin. Remarkably, a strong correlation between enzyme binding and the syringyl/guaiacyl (S/G) ratio was found for the lignins, which presented a similar hydroxyl group content (^{31}P NMR): higher protein affinity was determined on isolated spruce lignin (99% G units), while the lowest adsorption occurred on isolated eucalyptus lignin (70% S units). The effect of electrostatic interactions in enzyme adsorption was investigated by SPR, which clearly indicated that the screening of charges allowed more extensive protein adsorption. Overall, this work furthers our understanding of lignin−cellulase interactions relevant to biomass that has been subjected to no or little pretreatment and highlights the widely contrasting effects of the nature of lignin, which gives guidance to improve lignocellulosic saccharification and related processes.

INTRODUCTION

The replacement of nonrenewable resources in the production of chemical building blocks and liquid fuels has intensified research in the bioconversion area. The biorefinery concept aims to utilize lignocellulosics toward bioproducts, bioenergy, and high value streams for optimal utilization of nonfood biomass resources, widely available in the form of agricultural and forestry residues or from dedicated energy crops. A significant limitation in related efforts is the recalcitrance of lignocellulosic in bioconversion, which impacts severely the cost-effectiveness of biorefineries.[1,2] A typical lignocellulose bioconversion process involves multiple steps including physical, chemical, or biological pretreatments, enzymatic hydrolysis of cellulose, or the residual hemicelluloses into

Received: January 14, 2017
Revised: March 12, 2017
Published: March 13, 2017

DOI: 10.1021/acs.biomac.7b00071
Biomacromolecules 2017, 18, 1322−1332

sugars and fermentation.[3,4] The effectiveness of these processes is critically affected by the type, distribution, and chemical nature of the residual lignin.[5]

High value applications of lignin have been limited by the structural complexity of the molecule and its variability; thus, they are mostly utilized in energy cogeneration.[6] This is related to the fact that the main functions of lignin in nature is to provide microbial resistance and structural strength to the plant.[7] Lignin is synthesized by chemical polymerization of three main precursors: coniferyl, sinapyl, and *p*-coumaryl alcohols. These monolignols produce, respectively, guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) lignin units, which are incorporated in the macromolecule. The concentration of each of these units is highly variable and depends on the botanical origin of the plant. Softwoods, for instance, are made mostly of G units and small amounts of H units, while hardwoods are composed of both S and G units in different relative amounts. Grasses typically comprise the three units.[8] Some pretreatment methods partially dissolve lignin or rearrange it in different fragments that remain in the biomass, while other, less severe methods may preserve lignin in a state that is close to its native form.

Cellulases, which comprise a set of enzymes, act synergistically, among others, to break down the β-1,4 glycosidic bond that holds together the glucose units in cellulose. These enzymes are used in commercial mixtures to hydrolyze cellulose. They contain cellobiohydrolases (CBH) that hydrolyze the cellulose chains and liberate cellobiose from the reducing and the nonreducing ends. The mixtures also contain endoglucanases (EG), which act on linkages in the amorphous regions of cellulose chains and form new chain ends and oligomers. They release low molecular weight oligomers and cellobiose that are further hydrolyzed into glucose units by β-glucosidases.[9] The main enzyme in commercial mixtures of cellulases produced by *Trichoderma reesei* TrCel7A (CBH I, EC 3.2.1.176) is an exoacting cellulase that hydrolyses the ends of cellulose chain from the reducing end. For complete saccharification of cellulose within a reasonable time, relatively high enzyme loadings are needed, which make the process costly.[10] Thus, recent efforts have been directed to enhance the enzyme activity in economically viable processes.[11,12]

Lignin content in biomass and its detailed composition play critical roles in the saccharification of lignocellulosic materials, usually in decreasing the performance and efficiency of enzymatic hydrolysis. Several mechanisms of inhibition have been reported. For example, lignin may act as a physical barrier that restricts the access of the cellulases to cellulose, namely steric hindrance, or may adsorb cellulases, resulting in nonproductive binding.[3,13] Hydrogen bonding and hydrophobic and electrostatic interactions have been hypothesized to participate in nonspecific adsorption.[14,15] However, enzyme inhibition activity and related mechanisms remain untapped for elucidation to advance enzymatic hydrolysis processes.[13,16−18] Quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) techniques combined with model films of isolated lignins have been useful for this purpose.[13,17,19−21] For example, isolated lignin films have been prepared directly by drying from solution, by using the Langmuir−Blodgett technique[22−25] or by spin coating,[6,21,25,26] and several studies have addressed the interactions between lignin and different types of proteins (see for example our previous studies[13,19,27,28]). The control of the thickness and roughness the model films is essential to study lignin−cellulase interactions. In fact, the surface properties of the lignin films depend on the type of lignin, the method used for its extraction, and the technique used for film preparation.[20] Previous studies of lignin−cellulase adsorption were performed on isolated lignins including protease-treated lignin (PTL) and cellulolytic enzymatic lignin (CEL),[29] enzymatic mild acidolysis lignin (EMAL), and lignin-rich enzymatic hydrolysis residues (EnzHR).[17] These and other studies indicate different adsorption capacity depending on the lignin type.[19]

Commonly, pretreatments are necessary to increase the accessibility of enzymes to biomass. Such steps modify the lignin structure in various, complex ways. Consequently, differences are expected for the adsorption of cellulases on residual and native lignins. Extensive research, including our own,[17] has attempted to address the first case, while the present work focuses on the more native forms of lignins. Indeed, despite the many related seminal efforts, no reports exist in the context of cellulase binding on milled wood (MWL) or Björkman lignins,[30] which are considered close versions of the corresponding native structures.[31−34] Therefore, in this contribution we elucidate the hydrophobic and electrostatic interactions between isolated MWL and cellulases by using electroacoustic and optical techniques capable of sensing the extent and dynamics of the interactions, namely, the quartz crystal microgravimetry (QCM) and the surface plasmon resonance (SPR), respectively.

MATERIALS AND METHODS

Lignins were extracted from *Eucalyptus globulus* wood (ENCE, Pontevedra, Spain), wheat straw (Spain), and spruce wood (Sweden) according to the traditional Björkman procedure.[30] Briefly, ∼40 g of extractive-free material was finely ball-milled in a Retsch PM100 planetary ball mill (Restch, Haan, Germany) at 400 rpm using a 500 mL agate vessel with 20 agate ball-bearings (20 mm diameter). The total ball-milling time was 24 h, carried out by using 15 min on and off cycles. The ball-milled materials were then extracted with dioxane/water 96:4 (v/v), and the respective isolated crude MWL was then subsequently purified, as described elsewhere.[35] Thereafter, for simplicity, these milled wood lignins are referred to as MWL. All other solvents were of analytical grade.

Studies on enzyme adsorption were carried out with a purified *Trichoderma reesei* cellobiohydrolase TrCel7A (CBH I) as well as a commercial enzyme mixture. The cellobiohydrolase TrCel7A is an exocellulase that was purified according to Suurnäkki et al.[36] except for the omission of the last step of hydrophobic interaction chromatography on phenyl sepharose. The commercial cellulase used was under trade name CTec2 (Novozymes A/S, Denmark), a multicomponent enzymes based on the *T. reesei*. Naturally, the exact composition of this commercial mixture is not disclosed; such system is used here simply as a reference of industrial relevance. It can be assumed, however, that Ctec2 is an augmented mixture of proteins consisting of xylanases, endoglucanases, beta-glucanases, and other proteins.

MWL Characterization. *Lignin Content.* Klason lignin was estimated as the residue after sulfuric acid hydrolysis of the purified MWL, according to Tappi Standard T222 om-88.[37] The acid-soluble lignin content was determined by spectrophotometry (205 nm, extinction coefficient of 110 L/cm/g) according to Tappi Standard UM-250.[37] Besides these components, it is worth noting the possible contributions of carbohydrates and minerals. Carbohydrates, for instance, are always present in MWL preparations in the form of lignin−carbohydrate complexes (LCC).

Two-Dimensional Nuclear Magnetic Resonance Spectroscopy. For the 2D-NMR analysis, 30 mg of MWL was dissolved in 0.75 mL of deuterated dimethyl sulfoxide (DMSO-d_6). The heteronuclear single quantum correlation (HSQC) spectra were recorded at 300 K on a Bruker AVANCE III 500 MHz spectrometer (Bruker, Karlsruhe, Germany), equipped with a cryogenically cooled 5 mm TCI gradient

DOI: 10.1021/acs.biomac.7b00071
Biomacromolecules 2017, 18, 1322−1332

probe with inverse geometry (proton coils closest to the sample). The 2D $^{13}C-^{1}H$ correlation spectra were obtained using an adiabatic HSQC pulse program (Bruker standard pulse sequence 'hsqcetgpsisp2.2'). The spectral width was from 10 to 0 ppm for the ^{1}H dimension, with an acquisition time of 145 ms, and a recycle delay (d1) of 1 s. For the ^{13}C dimension, the spectral width was from 165 to 0 ppm, collected in 256 increments of 32 scans for a total acquisition time of 2 h 40 min. The $^{1}J_{CH}$ used was 145 Hz. Processing used typical matched Gaussian apodization in ^{1}H and a squared cosine bell in ^{13}C. The central solvent peak was used as an internal reference (δ_C 39.5; δ_H 2.49 ppm).

2D NMR HSQC cross-signals were assigned after comparison with data from literature.[38−40] A semiquantitative analysis of the volume integrals of the HSQC correlation peaks was performed using Bruker's Topspin3.1 processing software, according to previous studies.[38] The integration of the cross-signals was performed separately for the different regions of the HSQC spectrum, which contain signals that correspond to chemically analogous carbon−proton pairs. For these signals, the $^{1}J_{CH}$ coupling value is similar, and integrals can be used semiquantitatively to estimate the relative abundance of the different species. In the aliphatic oxygenated region, the relative abundances of side-chains involved in interunit linkages were estimated from the C_α/H_α correlations, except for substructures Aox and I, for which C_β/H_β and C_γ/H_γ correlations were used, respectively. In the aromatic region, C_2/H_2 and C_6/H_6 correlations from H, G, and S lignin units and from *p*-coumarate were used to estimate their relative abundances.

^{31}P Nuclear Magnetic Resonance. Methylation and oxypropylation of the MWLs were analyzed by quantitative ^{31}P NMR. Around 40−45 mg of dried isolated lignin was dissolved in 500 μL of a mixture of anhydrous pyridine/CDCl3 (1.6:1, v/v). A volume of 200 μL of an endo-*N*-hydroxy-5- norbornene-2,3-dicarboximide solution (9.2 mg/mL) was used as internal standard, and 50 μL of a chromium(III) acetylacetonate solution (5.6 mg/mL), used as relaxation reagent, was added. Finally, 100 μL of phosphorylating reagent II (2-chloro-4,4,5,5-tetramethyl-1,2,3- dioxaphospholane) was added and transferred into a 5 mm NMR tube for subsequent NMR acquisition. NMR spectra were acquired using a Bruker 300 MHz spectrometer equipped with a Quad probe dedicated to ^{31}P, ^{13}C, ^{19}F, and ^{1}H NMR acquisition.

Electrophoretic Mobility. The electrophoretic mobility, evaluated here as the zeta potential, of the MWLs at different buffer concentrations and pH was measured. Prior to the measurements, the lignins were mixed in buffer solutions (50 mL) by using a shaker/incubator at 50 °C and 200 rpm.[41] Acetate buffer solutions of 50, 100, 200, and 500 mM concentration at pH 5.2 were used. Acetate buffers at pH 3.3, and 6.5 at 200 mM were also applied. The lignin concentration in all the buffer solutions was 0.033% (w/v). The zeta potential was measured on the supernatant of the dispersions by using a Zetasizer Nano series (Nano ZS, Malvern, UK). All measurements were performed in triplicate with three readings each. The size of the lignin colloids was determined at 100 mM concentration in buffer solution at pH 5.2, 25 °C. A refractive index of 1.6 was assumed for the lignins,[16] while that for the enzymes was assumed to be 1.45.[42]

Spin-Coated MWL Thin Films. A 0.5 wt % MWL lignin solution was prepared by dissolving MWL in 1,4-dioxane 2 days prior to use. The supernatant of the dispersion was used to produce the spin-coated films (spin-coater from Laurell Technologies Corporation WS-400A-6NPP/LITE, USA.). Prior to spin coating, silica (AFM) or gold-coated quartz sensors (QCM, SPR) were cleaned by rinsing with ethanol and dried with nitrogen. Silicon wafers were also used as substrates (AFM, contact angle). They were cut in 1 × 1 cm^2 pieces and their surfaces activated by immersing them in a 1 M sodium hydroxide for 15 s.

For spin coating, the speed, time, and lignin concentration were optimized to produce films of reproducible and suitable thickness (2000 rpm for 20 s, 1750 $rpms^{-1}$ acceleration). Prior to the lignin deposition, the silica/gold surface was precoated with polystyrene (PS) dissolved in toluene (0.5 wt %) using same conditions as those for lignin. The sensors were dried at 80 °C for 30 min. For each sample, MWL was spin coated eight times. The films were stored in a desiccator until further used.

Characterization of Lignin Thin Films. AFM imaging was performed to assess the morphology and roughness of the lignin films. The lignin films were mounted on aluminum holders and examined with a Dimension 3000 scanning probe microscope from Veeco Metrology Group. Scanning was performed in air using the tapping mode with silicon cantilevers (NSCl5/AIBS) delivered by Olympus AC160TS. The drive frequency of the cantilever was about 275−325 kHz (nominal resonance of 300 kHz). The areas scanned included 200 × 200 nm^2 and 3 × 3 μm^2 sizes. No image processing except flattening was performed. Images were acquired with a feedback loop to keep the amplitude of oscillation constant and measured the response of the feedback loop. The response of the feedback loop was used to measure how far the scanner was moved in Z to keep the amplitude of oscillation constant.

Ellipsometry. A variable angle spectroscopic ellipsometer (J.A. Woollam) with coincident He−Ne laser and capable of spatial mapping of dielectric properties was used to determine the thicknesses of the prepared lignin model surfaces. The variation in optical properties over a large area, the distribution of the ξ (psi/delta) and ψ (wavelength) ellipsometric parameters, were measured over an area with approximately 2 mm sides. The thickness of the lignin film was determined from the measured ξ and ψ parameters as the angle of incidence was varied between 65° and 70°. The model assumed was air/lignin/PS/silica/silicon, and all materials were assumed to be optically isotropic. The refractive indices of the materials were assumed to be 3.5 (silicon) and 1.45 (silica). The calculations employed a least-squares iterative fitting procedure using both thickness and refractive index of the lignin and polystyrene layers as fitting parameters. The measurements were performed under ambient air and relative humidity conditions. The measurements were repeated several times at different positions on the surface and also with different surfaces that were prepared under the same conditions.

Contact Angle Measurements. The water contact angle on the lignin surfaces were measured using a video-controlled PHX 300 contact angle goniometer (Surface Electro Optics, Phoenix). The contact angles were determined by curve fitting of the drop shape the of ImageJ software.

Enzyme Adsorption Studied in Electroacoustic Experiments. Enzyme adsorption on the lignin films was followed with an E4 Quartz crystal microbalance (Q-Sense, Gothenburg, Sweden) operating in a continuous mode. The QCM-D was used to monitor the adsorption of CTec2 and CBH-I on the three different MWL films. CTec2 was used at 5 mg/mL, and CBH-I was applied at 1 mg/mL, the enzymes were diluted with freshly prepared 100 mM sodium acetate buffer at pH 5.2 (injection rate of 100 μL/min and 25 °C). The base signal was obtained, while the sensors were kept in buffer for 1 h. Once equilibrated, data acquisition was restarted, and the enzyme solution was introduced after 5 min. After a given time, enzyme-free buffer was injected for rinsing, and the signals were used to determine the level of reversible and irreversible adsorption. All measurements were recorded at 5 MHz fundamental resonance frequency and its overtones corresponding to 15, 25, 35, 55, and 75 MHz. The third, fifth, and seventh overtones were used for data processing. The Johannsmann method was used to calculate the mass of enzyme adsorbed on the surface of the sensor.[43]

Enzyme Adsorption via Surface Plasmon Resonance. The effect of the electrostatic interactions between the enzymes and lignin was investigated with a multi-parametric surface plasmon resonance unit (MP-SPR Model Navi 200, BioNavis Oy Ltd., Ylöjärvi, Finland) operated at fixed angle mode. The experiments were carried out at 25 °C, with a flow rate of 50 μL/min. The concentration of sodium acetate buffer at pH 5.2 was varied between 50 mM and 200 mM. The amount of protein absorbed on the lignin per unit area, Γ, was calculated using eqs 1 and 2:

$$d = \frac{l_d}{2}\frac{\Delta\theta}{m(\eta_a - \eta_o)} \quad (1)$$

$$\Gamma = \rho d \quad (2)$$

DOI: 10.1021/acs.biomac.7b00071
Biomacromolecules 2017, 18, 1322−1332

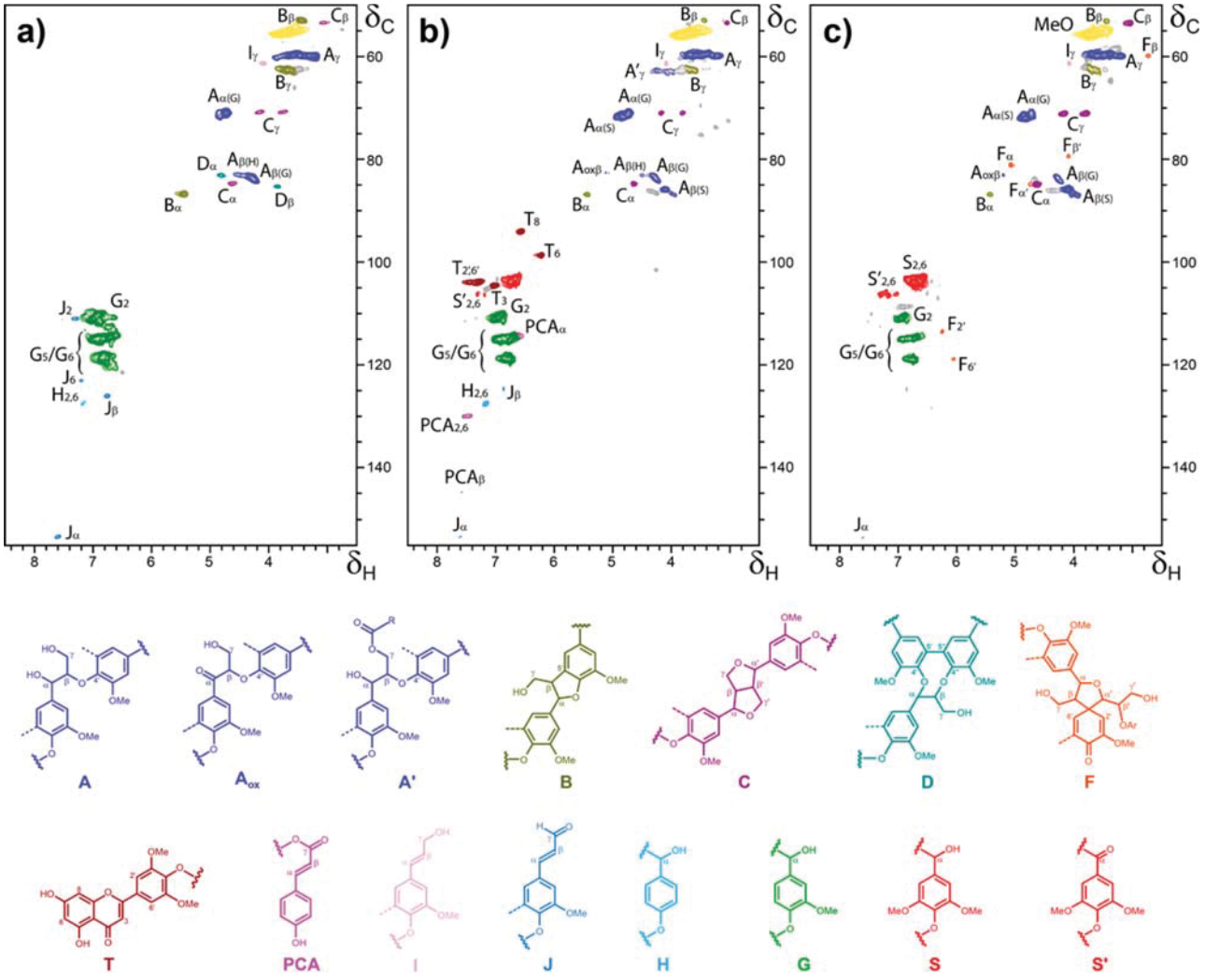


Figure 1. Heteronuclear single quantum correlation nuclear magnetic resonance spectra of (a) spruce, (b) wheat straw, and (c) eucalyptus isolated as milled wood lignins (MWL) with main structures identified. See Table S1 for quantification of the structures.

where d is the thickness of adsorbed layer, $\Delta\theta$ is the angle shift, l_d is a characteristic evanescent electromagnetic field decay, estimated to be ~0.37-times the wavelength of the incident light (240 nm),[44] m is a sensitivity factor for the sensor (109.95°/RIU, RIU: refractive index units) obtained by calculating the slope of a $\Delta\theta$ calibration with solutions of known refractive indices,[45] η_o is the refractive index of the background solution (buffer, 1.3342), and η_a is the refractive index of the adsorbed species (enzyme), which was assumed to be 1.57. ρ is the bulk density of the enzyme (1370 kg/m^3) and was determined from specific volume data (0.73 mL/g).

RESULTS AND DISCUSSION

Milled Wood Lignins (MWL). The concentration of acid soluble lignin, ASL, was 2.3, 1.2, and 0.5% for spruce, wheat straw, and eucalyptus, respectively. The total lignin concentration (expressed as Klason plus ASL) was quite similar for the three types of MWL, between 85 and 87%. The S/G ratio of the samples increased with the ASL, but the main differences are further shown with respect to phenolic hydroxyl, carboxylic acid, and aliphatic hydroxyls groups. Moreover, 2D-NMR was used to access the detailed composition of the MWL samples and their interunit linkages. The main lignin cross-signals assigned in the 2D HSQC spectra of the MWL isolated from eucalyptus, wheat straw, and spruce (Figure 1) are listed in Table S1 of the Supporting Information. The spectra indicated major differences between the isolated MWL, in terms of composition and linkages: see Table 1 for the relative abundances of the main interunit linkages and end-groups, as well as the percentage of γ-acylation, the molar abundances of the different lignin units (H, G and S), p-coumarates, and the molar S/G ratios of the MWL extracted, which were estimated from volume integration of contours in the HSQC spectra.

The MWL from spruce, as a coniferous species, almost exclusively comprised G-lignin units (99%), with a very low amount of H units (1%). In contrast, eucalyptus MWL was mainly composed of S-lignin units (69%), with lower amount of G lignin (31%) and a S/G ratio of 2.1. The MWL extracted from wheat straw was rich in G units and had a S/G ratio of 0.5 (H/G/S = 3:61:36). The relative abundance of the different interunit linkages reflected the compositional differences noted for the three extracted MWLs. Although, β-O-4′ linkages were most abundant in all of these lignins, important amounts of β-5′ (phenylcoumaran) and 5−5′ (dibenzodioxocin), which are related to the presence of G lignin units, were also found in the MWL extracted from spruce. Interestingly, MWL extracted

DOI: 10.1021/acs.biomac.7b00071
Biomacromolecules 2017, 18, 1322−1332

Table 1. Structural Characteristic Lignin Interunit Linkages, End-Groups, γ-Acylation, Aromatic Units, S/G Ratio, and Cinnamate Content Obtained from Integration of $^{13}C-^{1}H$ Correlation Peaks in the HSQC Spectra of the MWL Isolated from Eucalyptus, Wheat Straw, and Spruce

	eucalyptus	wheat straw	spruce
Lignin Interunit Linkages (%)			
β−O−4′ aryl ethers (A/A′)	80	78	62
α-oxidized β−O−4′ aryl ethers (Aox)	3	2	0
phenylcoumarans (B)	4	9	25
resinols (C)	9	10	5
dibenzodioxocins (D)	0	1	8
spirodienones (F)	4	0	0
Lignin End-Groups			
Cinnamyl alcohol end-groups (I)[a]	6	5	4
Cinnamaldehyde end-groups (J)[a]	0	3	4
Lignin side-chain γ-acylation (%)	0	12	0
Lignin Units (%) and S/G Ratio			
S	67	36	0
G	33	61	99
H	0	3	1
S/G ratio	2.0	0.5	0
p-coumarates (%)[b]	0	4	0

[a]Expressed as a fraction of the total lignin interunit linkage types **A**−**F**. [b]*p*-Coumarate molar content reported as percentage of lignin content (S + G + H).

from wheat straw was acylated at the gamma position of the side chains of *p*-coumarate and presented important amounts of tricin, as previously reported.[38]

The hydroxyl group content of the different MWL was determined by ^{31}P NMR spectroscopy (see Figure 2 for the ^{31}P NMR spectra of the MWL). The results obtained from ^{31}P NMR are included in Table 2 and indicate that the amount of total hydroxyl groups was similar for all the MWL studied. The MWL extracted from wheat straw and eucalyptus presented a relatively higher phenolic hydroxyl group content, whereas the MWL from spruce was enriched in aliphatic hydroxyl groups. Since the total hydroxyl group content was not too different in the isolated lignins, our studies about the adsorption of cellulases on lignins were mainly concerned with the effect of lignin composition, for example, in terms of the S/G ratio.

Zeta Potential of Lignins and Cellulases. In addition to the effects of molecular composition, the interactions between lignins and cellulase enzymes depend on electrostatic interactions in aqueous media, which in turn depend on the charge balance (zeta potential) of the dispersed MWLs (Figure 3a) and enzymes (Figure 3b). All the MWLs were negatively charged at pH 5.2, as expected; in all the cases, also as expected, the charge was reduced with the increase in ionic strength due electrostatic screening effects. Table S2 of Supporting Information provides additional data. As noted in Figure 3a, in the range between 50 and 100 mM, the zeta potential was between −23 and −17 mV, while it was reduced to −12 and −6 mV for ionic strength of 200 mM and 500 mM, respectively.

The molecular mass (MALDI-ToF) and isoelectric point (pI) of the CBH-I were 56 kDa and 3.82, respectively, in agreement with other reports.[46,47] Compared with the lignins, the enzymes displayed a lower negative charge at pH = 5.2 (Figure 3b).

Ultrathin Films of the MWLs. AFM height images of thin films obtained by spin-coating on polystyrene of MWL

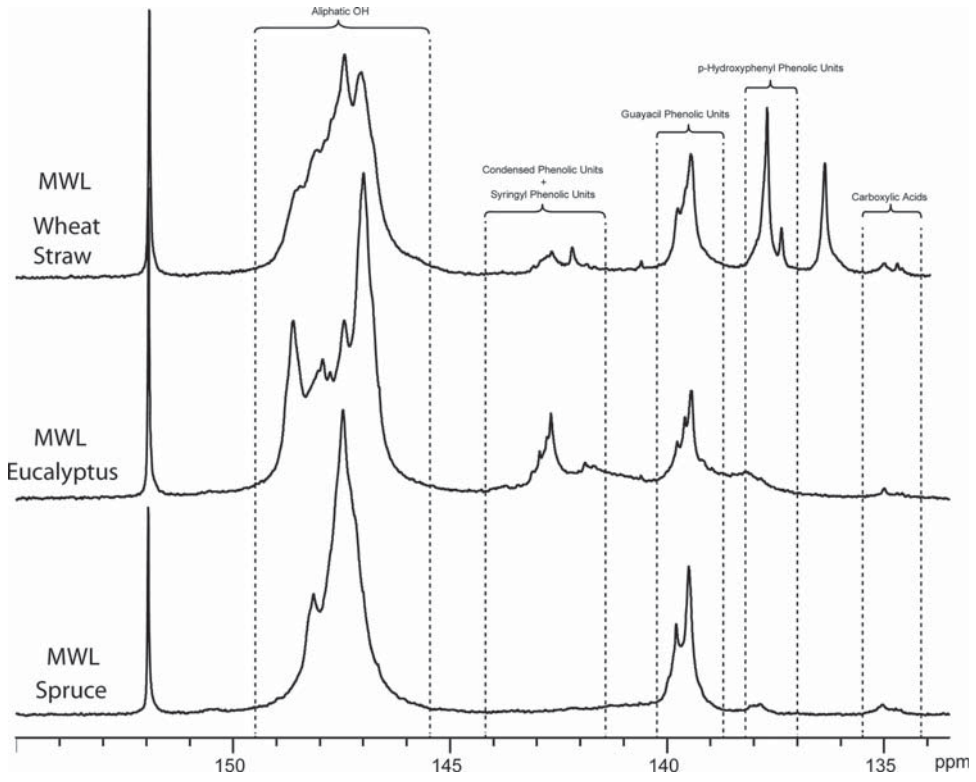


Figure 2. ^{31}P NMR spectra of milled wood lignins isolated from wheat straw, eucalyptus, and spruce, as indicated.

DOI: 10.1021/acs.biomac.7b00071
Biomacromolecules 2017, 18, 1322−1332

Table 2. Compositional Analysis from ^{31}P NMR Spectra of MWL Extracted from the Lignocellulosic Sources Studied, as Indicated[a]

sample	aliphatic OH	phenolic OH	S+C	G	H	COOH	total OH
spruce	5.40	1.51	0.26	1.18	0.07	0.03	6.95
wheat straw	4.72	2.06	0.54	0.92	0.63	0.08	6.89
eucalyptus	4.91	2.09	1.12	0.63	0.27	0.02	7.02

[a]Data expressed as mmol/g.

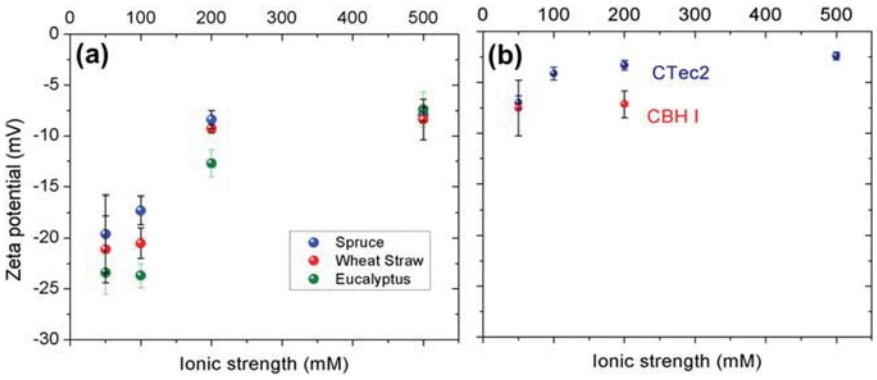


Figure 3. Surface charge reported as zeta potential and measured in aqueous dispersions of given ionic strength and pH = 5.2 for (a) MWL samples: spruce (blue), wheat straw (red), and eucalyptus (green). (b) Also plotted is the zeta potential for the enzymes (CBH-I and CTec2).

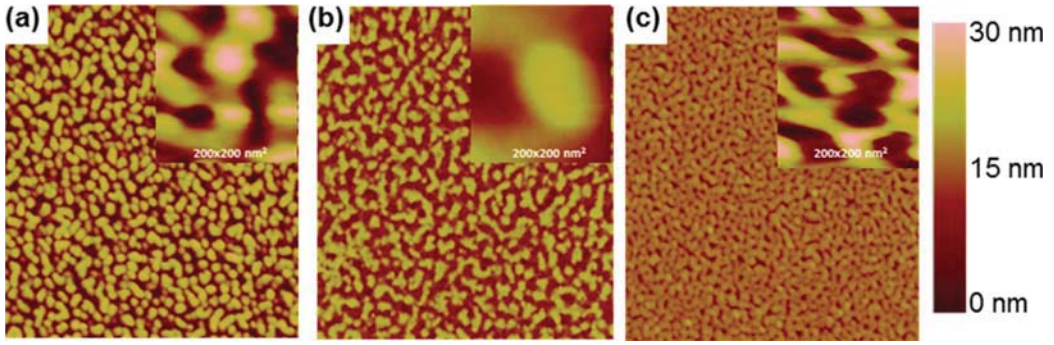


Figure 4. AFM topography (3 × 3 μm^2 images) of MWL films spin coated on polystyrene. (a) Spruce, (b) wheat straw, and (c) eucalyptus. The insets include images at higher magnification 200 × 200 nm^2.

extracted from spruce, wheat straw, and eucalyptus are shown in Figure 4. The respective root−mean−square (RMS) AFM roughness measured for at least four images were 5.41 ± 0.16, 4.15 ± 0.03, and 2.14 ± 0.04 nm (note the roughness measured for the PS support was 0.3 nm). Spherical features, with sizes in the range of 10−20 nm, were observed in the AFM images. The morphology of the surfaces was characteristic of other lignin films prepared by the spin-coating technique.[6] Images acquired at lower magnification indicated that the films were continuous over large areas (images not shown).

The RMS roughness of MWL films varied considerably depending on the method used to isolate the lignin.[48] The possibility to control the thickness and roughness of lignin films is critical to study the interactions between lignin and cellulases. Indeed, smooth films are preferred when techniques such as QCM are used.[34] From the images and RMS roughness values, it is concluded that the lignin films fully covered the surface and were relatively smooth. However, it is interesting to note that the spruce and wheat straw films were slightly rougher compared to the films obtained from eucalyptus MWL. It is possible that the more significant presence of syringyl units in the latter resulted in smoother lignin films. This is because the positions 3 and 5 of the aromatic rings are blocked by methoxyl groups, which translates into more linear lignin structures.

The thickness of the prepared lignin thin films was determined by ellipsometry: 14, 9, and 9 nm for spruce, wheat straw, and eucalyptus MWL, respectively. Interestingly, the film roughness remained relatively low independent of the film thickness. On the basis of fitted data from more than 50 ellipsometry measurements, the refractive index was determined to be μ = 1.60 ± 0.03 for the MWLs studied, similar to the values reported by Norgren et al.,[6] for kraft lignins (μ = 1.61 ± 0.04). However, they were larger than those for lignins extracted, via acetosolv or organosolv methods, from sugar cane bagasse and from *Pinus caribaea var. hondurensis* (μ = 1.04−1.30 for 350−800 nm wavelength).[23] The porosity of these latter lignin films was lower, which yielded denser films. Interestingly, the roughness remained relatively low independent of the film thickness.

Cellulase Binding on MWL Films. Images of sessile water droplets in contact with the MWL films, taken before and after CBH-1 adsorption, revealed a clear reduction in contact angle,

DOI: 10.1021/acs.biomac.7b00071
Biomacromolecules 2017, 18, 1322−1332

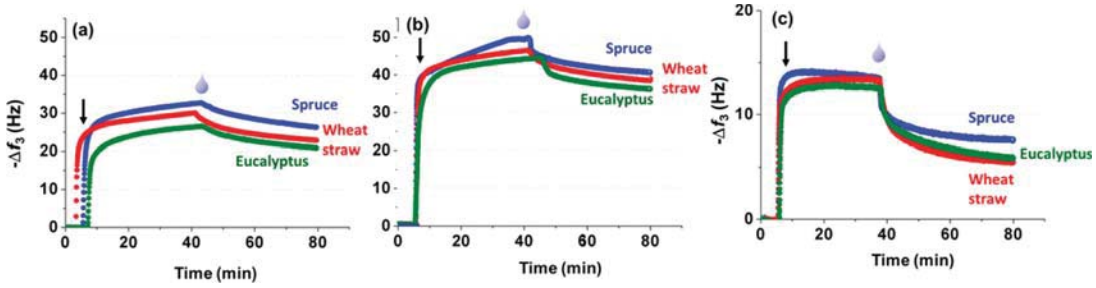


Figure 5. Quartz crystal microgravimetry (QCM) sensograms upon injection of CTec2 on MWL thin films. Two different enzyme concentrations were used, (a) 1 and (b) 5 mg/mL. (c) Included is also the sensogram for CBH-I added at 1 mg/mL concentration. In each figure, the arrow on the left indicates the approximated time at which enzyme was injected after film equilibration in background electrolyte, and the "drop" symbol represents the time at which the enzyme solution was replaced with background electrolyte solution (rinsing step). The source of the MWL used in film preparation is indicated (spruce, wheat straw, and eucalyptus).

Table 3. Apparent QCM Mass of Enzyme (CTec2 or CBH-I) Adsorbed on Thin MWL Films As Measured by the Respective Sensograms[a]

	CTec2 (1 mg/mL)		CTec2 (5 mg/mL)		CBH-I (1 mg/mL)	
MWL	reversible	irreversible	reversible	irreversible	reversible	irreversible
spruce	5.4 ± 0.4	4.3 ± 0.7 (80%)	7.9 ± 0.9	6.2 ± 0.9 (79%)	2.5 ± 0.3	1.3 ± 0.2 (52%)
wheat	4.9 ± 0.3	3.7 ± 0.1 (76%)	7.5 ± 0.4	6.0 ± 0.3 (80%)	2.4 ± 0.1	1.1 ± 0.3 (46%)
eucalyptus	4.3 ± 0.5	3.3 ± 0.3 (77%)	7.2 ± 0.4	5.7 ± 0.3 (79%)	2.2 ± 0.4	1.0 ± 0.1 (45%)

[a]See Figure 5 with examples in the case of CTec2 introduced at two concentrations. The data are reported in units of mas per unit area, mg/m^2. The reversible adsorbed mass calculated upon enzyme injection is determined after the frequency signals reaches equilibrium (about 40 min after injection). The mass determined from frequency values obtained after rinsing with background electrolyte is taken as the enzyme that is irreversibly adsorbed on the substrate.

from 66–69 degrees (depending on lignin source) to 30–40 degrees (Figure S1 and Table S3). This was a consequence of enzyme adsorption on the surface, as will be explained in more detail with QCM experiments, whereby hydrophobic amino acids of the cellulases interacted with the lignin, which left exposed hydrophilic residues of the enzymes.

Two different concentrations of CTec2 were used in experiments to reveal the binding and dynamics of enzyme adsorption on the lignin-coated QCM sensors (Figure 5a and b for 1 and 5 mg/mL CTec2). An increase in the negative value of the frequency shift, $-\Delta f_3$, indicated mass uptake or adsorption onto the film. After the baseline acquired in the background electrolyte and upon introduction of the enzyme (in the same electrolyte solution, left arrow to indicate approximate time of injection), an increase in the adsorbed mass (increase in $-\Delta f_3$) was noted for all the lignin films. The rate at which the enzyme was adsorbed can be taken as indicative of the early stages of the adsorption process. Only small differences were observed if one compares the adsorption profiles on the different MWL films, Figure 5a and b. The enzymes adsorbed to a larger extent onto spruce MWL, but the opposite was observed for eucalyptus MWL. The apparent mass of enzyme (CTec2 and CBH-I) adsorbed on the different MWL films, calculated from QCM sensograms, is reported in Table 3. Enzyme adsorption increased with equilibrium concentration (isotherms acquired at other concentrations, not shown) but only to a limited extent, which indicated the possibility that surface saturation was nearly reached at 5 mg/mL solution concentration.

As far as the interactions between lignin and Ctec2, we would like to point out that given the complex mixture of proteins that exist in this commercial system, caution must be exercised in any effort to correlate the binding signature with that of purified CBH1. For this purpose, other enzyme cocktails known to comprise enzyme preparations from *T. reesei*, and that may not be augmented, may be more relevant. However, Ctec2 is simply taken here as an example of a commercial system that is widely reported and used for total hydrolysis. Here, we attempted to identify any significant differences in the lignin-binding behavior, which as indicated by the data seems to be sensitive to the exact composition of the reference enzymes.

Rinsing with background buffer was carried out to determine the extent at which lignin was removed and thus to gain some understanding on the irreversibility of the adsorption process. The adsorbed mass was calculated by using the Johannsmann method[50] from QCM frequency values. In the calculation, the density of the AT-cut quartz crystals, ρ_q, was assumed to be 2648 kg/m^3. The shear modulus, μ_q, was assumed to be 2.95×10^{10} kg/m^2. The third, fifth, and seventh overtones of the resonance frequencies were used to calculate the mass m_i at each frequency (i = 3, 5, or 7) using eq 3:

$$m_i = \frac{\sqrt{\rho_q \mu_q}\,\Delta f}{2 f_i} \tag{3}$$

m_i was plotted as a function of the square of the resonance frequency, and the mass of the adsorbed layer was determined by extrapolation at a resonance frequency zero. The frequencies of the QCM sensor were measured in buffer before the deposition of polystyrene and lignin. The obtained values of adsorbed mass, at 40 min and after rinsing at 80 min, for the reversible and irreversible adsorption, respectively, are included in Table 3. The percentage of enzyme that irreversibly adsorbed onto lignin was noted to be similar for both of the enzyme doses investigated. Please note that as any other approach to calculate the adsorbed mass, the Johannsmann model is subject to assumptions and can be only taken on a relative basis.

DOI: 10.1021/acs.biomac.7b00071
Biomacromolecules 2017, 18, 1322–1332

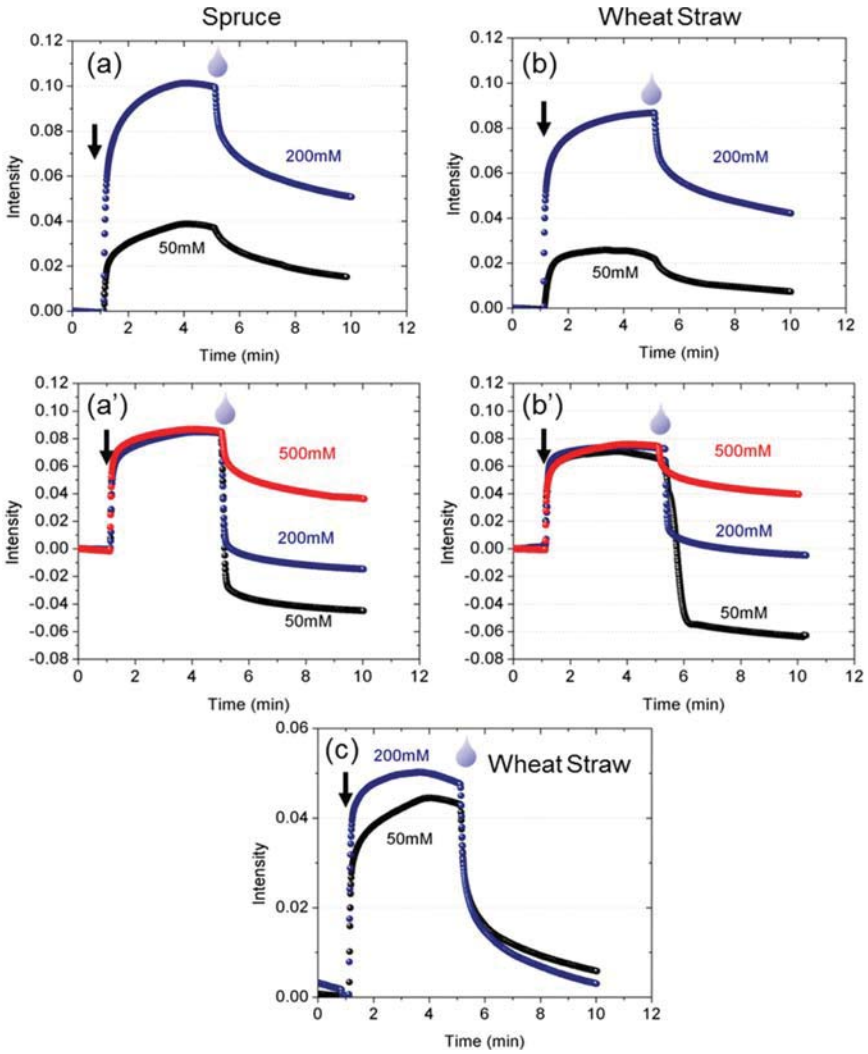


Figure 6. SPR sensograms upon adsorption of CTec2 (5 mg/mL) dissolved in aqueous solution of 50 and 500 mM electrolyte concentration on (a) spruce MWL and (b) wheat straw MWL. In these cases, rinsing was performed with enzyme-free, electrolyte solution of the same concentration. Additional experiments were conducted by introducing the enzyme in 500 mM background electrolyte concentration followed by rinsing with electrolyte solutions of given concentrations (50, 200, and 500 mM): (a′) on spruce MWL and (b′) on wheat straw MWL. Experiments similar to (a) and (b) are shown in (c) and (d) for CBH-I adsorption on wheat straw MWL. In each figure, the arrow symbol represents the approximated time at which enzyme was injected after film equilibration in background electrolyte, and the "drop" symbol represents the time at which the enzyme solution was replaced with background electrolyte solution of given concentration, as indicated (rinsing).

Enzyme adsorption has been indicated to scale with both the S/G ratio and the amount of phenolic and aliphatic hydroxyl groups.[17,49,51] For the MWLs studied here, the concentration of total hydroxyl groups was very similar (^{31}P NMR analysis), while there was significant difference in the S/G ratio (2D NMR data). Thus, considering the extent of enzyme adsorption, there was clear indication that it increased with the G content of the lignin.

The presence of surfactants and preservatives in commercial enzyme cocktails influences the interactions between lignin and cellulase.[52] However, in the present case, no attempt was made to remove (via solvent exchange, etc.) surfactants or preservatives for two reasons: first, the effects were expected to be small given the levels of dilution, and second, the system was tested in conditions relevant to actual applications.

Data from the purified enzyme are discussed now in light of the adsorption experiments. The monocomponent cellulase from *T. reesei* Cel7A (CBH-I) was a cellobiohidrolase that represents about 50−60% of total enzyme cocktails produced by *T. reesei*. The function of this enzyme is to break down cellulose by an exo mechanism from the reducing ends. The extent of adsorption of the single component enzyme on the lignin films (Figure 5c) was distinctively less than that measured for the commercial enzyme mixture (Figure 5a). Compared to the case of the enzyme mixture, CBH-I followed the same adsorption trend on the three MWLs. The amount of CBH-I irreversibly adsorbed on MWL accounted for about half of the total mass adsorbed at equilibrium after injection. An interesting observation was that compared to the two other substrates, adsorption of CBH-I on spruce MWL was faster (data for short adsorption times, not included). One noticeable fact is that the relative amount of enzyme irreversibly adsorbed on spruce MWL was about 80% for CTec2, while that for CBH-I was 52%. These figures were 45 and 46% in the case of

DOI: 10.1021/acs.biomac.7b00071
Biomacromolecules 2017, 18, 1322−1332

eucalyptus and wheat straw MWL, respectively. Thus, the monocomponent enzyme adsorption on lignin was more limited.

Electrostatic and Rinsing Effects Revealed by SPR. Electrostatic effects are known to affect the interactions between lignin and enzymes.[53] Here, we discuss experiments carried out at pH 5.2 and different ionic strengths by the optical technique, surface plasmon resonance (SPR). For this purpose, wheat straw and spruce lignin were compared since they present the largest differences in lignin composition. In the experiments, enzyme was first adsorbed for 4 min, and then the surface was rinsed with background buffer. After 5 min, data were acquired to study the irreversibility of enzyme binding to lignin (SPR sensograms in Figure 6).

Enzyme adsorption experiments were carried out in buffer solutions of 50 and 200 mM salt concentration since, as shown in Figure 3, an important difference in lignin zeta potential was determined in these conditions (see also Table S2 with the zeta potential of MWLs at different ionic strengths). Compared to the case of low (50 mM) background buffer concentration, at 200 mM (when lignin is less negative) adsorption occurred to a larger extent, Figure 6a and b. At the higher ionic strength, the salts screened the electrostatic repulsion between the negatively charge enzymes allowing for better binding. Results for adsorption of the enzyme mixture, CTec2, are shown in Table 4. It is apparent that under similar conditions SPR adsorbed mass (Table 4) was smaller than that calculated from QCM sensograms (Table 3). This is explained by the fact that QCM is sensitive to hydration or coupled water. Also, SPR revealed, as was the case of QCM data, that adsorption on wheat straw lignin was higher for the enzyme cocktail compared to the monocomponent CBH-I. More important to the present discussion is that the irreversible adsorption of enzyme was significantly affected by the ionic strength: for spruce MWL, the relative fraction of enzyme that was irreversibly adsorbed was 38% at 50 mM, and it increased to 50% at 200 mM. The same applied to wheat straw MWL (33 and 50%, respectively). Thus, adsorption was favored under conditions of reduced electrostatic repulsion.

Table 4. CTec2 Adsorbed Mass (mg/m^2) Determined by SPR on Spruce and Wheat Straw MWL[a]

		(a) CTec2 SPR adsorbed mass at given background electrolyte conc.		(b) CTec2 SPR mass adsorbed from 500 mM after rinsing with given electrolyte conc.			(c) CBH-I SPR adsorbed mass at given background electrolyte conc.	
		50 mM	200 mM	50 mM	200 mM	500 mM	50 mM	200 mM
spruce	rev.	1.6	4.2			3.4		
	irrev.	0.6	2.1	3.3	3.1	1.4		
wheat straw	rev.	0.9	3.2			2.8	1.4	1.6
	irrev.	0.3	1.6	2.6	2.8	1.5	~ 0	0.2

[a]Measured upon adsorption at (a) 50 and 200 mM electrolyte concentration (see Figure 6a,b). (b) Adsorption level obtained from 500 mM electrolyte solution after rinsing with enzyme-free, electrolyte concentrations of 50, 200, and 500 mM, as indicated. (c) Mass adsorption (mg/m^2) for CBH-I is also included for conditions similar than those in panel a.

Experiments were conducted with enzymes in 500 mM background electrolyte concentration followed by rinsing with electrolyte solutions of given concentrations (50, 200, and 500 mM), Figure 6a′ and b′. The initial mass adsorbed was equivalent to ~3.3 mg/m^2 on spruce MWL and 2.7 mg/m^2 on wheat straw MWL. Upon rinsing with 500 mM buffer, the % fraction of the enzyme that was irreversibly adsorbed on spruce and wheat straw MWL were 41 and 54%, respectively. Results after rinsing with 200 and 50 mM electrolyte solutions are shown also in Figure 6a′ and b′. The total mass values on spruce and wheat straw MWL after rinsing with these solutions were less than the initial mass, which indicated partial removal of the lignin, possibly in the form of lignin−enzyme complexes. Indeed, AFM images obtained from the sensors for the experiments with 50 mM electrolyte concentration indicated partial removal of MWL, though some fragments still remained on the sensor, Figure S2. The roughness of the MWL substrate increased to 7 nm (spruce) and to 5.9 nm (wheat straw).

Proteins or enzymes undergo different adsorption mechanisms, which are influenced mainly by hydrophobic and electrostatic interactions between the proteins and the surface. Adsorption is possible to occur in the form of patches or uniformly distributed mono- or multilayers. The respective surface coverage of the enzymes on the surface films can be estimated from the adsorption data obtained via SPR. Three different scenarios were considered for this purpose, as shown in Figure S3. Accordingly, enzyme coverage on the MWL films was calculated for the different experiments and Table S4 shows the corresponding coverage values. There is an indication that patches are the primary enzyme arrangement on the surface, which was influenced by the structural conformations and charge of the enzymes. Compared to data obtained at high ionic strength, the electrostatic repulsions that existed in conditions of lower ionic strength were clearly shown as a decrease in enzyme coverage on the surface by more than 25%.

In concluding this discussion, it is important to point out a few items that are pertinent: residual lignins that result from biomass pretreatment (saccharification process, etc.) are most relevant as far as their interactions with enzymes. Such subject has been discussed, for example, in refs 17, 18, and many others, where the results for exo- and endoglucanases indicated different binding degrees. For example, exo- and endoglucanases showed very little affinity toward the lignin extracted from the pretreated corn stover, which is in contrast with the results presented in this work for the various milled wood lignins tested. In these latter cases, enzyme binding is a scientifically important aspect that, surprisingly, has received little attention. More importantly, the results clearly indicated that lignin−enzyme interactions depended on the nature of the substrates. While this was tested for different biomass sources, the same applied to other factors such as the level of pretreatment severity.

We should point out that the type of surfaces and the surface sensitive methods used here revealed fundamental aspects about the interactions that, nevertheless, are only approximations to actual process conditions, where mechanistic studies are not possible or very challenging. Indeed, the affinity of enzymes with lignin is affected by many physical properties of individual enzymes or enzyme types that influence adsorption rates and mechanisms. Moreover, such factors may not be displayed in mixed population of proteins, where the competitive binding shown by multiple enzymes for the same substrate may affect their interactions.

DOI: 10.1021/acs.biomac.7b00071
Biomacromolecules 2017, 18, 1322−1332

CONCLUSIONS

Multicomponent and a monocomponent *Trichoderma reesei* exoglucanase (CBH-I) enzymes adsorbed extensively on films of lignins isolated from spruce, wheat straw, and eucalyptus. Compared to the multicomponent cellulases, CBH-I displayed lower affinity with lignin and higher adsorption reversibility. These results challenge the standing assumption that this enzyme has a high affinity toward lignin and further highlight the relevance of different sources of lignins. QCM and SPR indicate that charge screening allows more extensive protein adsorption, which reveals the importance of electrostatic interactions in the mechanism of enzyme action in the presence of residual lignins. Finally, a correlation between the extent of adsorption and the S/G ratio of the lignins was found.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.7b00071.

Assignments of lignin $^{13}C/^{1}H$ correlation signals; size and zeta potential; water contact angles; AFM images; schematics of enzymes adsorption (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: orlando.rojas@aalto.fi. Phone: +358-(0)50 512 4227.

ORCID

Orlando J. Rojas: 0000-0003-4036-4020

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This study was partially funded by the INDOX EU-project (KBBE-2013-7-613549); the LIGNOCELL, LIGNIN, and BIORENZYMERY Spanish MICINN (cofinanced by FEDER funds) projects (AGL2011-25379, CTQ2014-60764-JIN, and AGL2014-53730-R); and the CSIC Project 201440E097. A.P. is grateful to the Spanish MINECO for a FPI fellowship. O.J.R. would like to thank the Academy of Finland for funding through its Centers of Excellence Program (2014−2019), under Project No. 132723612 HYBER.

REFERENCES

(1) Chundawat, S. P.; Beckham, G. T.; Himmel, M. E.; Dale, B. E. *Annu. Rev. Chem. Biomol. Eng.* **2011**, *2*, 121−145.

(2) Kumar, R.; Wyman, C. E. *Biotechnol. Bioeng.* **2014**, *111* (7), 1341−1353.

(3) Chandra, R. P.; Bura, R.; Mabee, W. E.; Berlin, A.; Pan, X.; Saddler, J. N. *Adv. Biochem. Eng./Biotechnol.* **2007**, *108*, 67−93.

(4) Mosier, N.; Wyman, C.; Dale, B.; Elander, R.; Lee, Y. Y.; Holtzapple, M.; Ladisch, M. *Bioresour. Technol.* **2005**, *96* (6), 673−686.

(5) Guo, F.; Shi, W.; Sun, W.; Li, X.; Wang, F.; Zhao, J.; Qu, Y. *Biotechnol. Biofuels* **2014**, *7* (1), 38.

(6) Norgren, M.; Notley, S. M.; Majtnerova, A.; Gellerstedt, G. *Langmuir* **2006**, *22* (3), 1209−1214.

(7) Sarkanen, K. V.; Ludwig, C. H. *Lignins: Occurrence, Formation, Structure, and Reactions*; Wiley-Interscience: New York, 1971.

(8) Boerjan, W.; Ralph, J.; Baucher, M. *Annu. Rev. Plant Biol.* **2003**, *54*, 519−546.

(9) Kumagai, A.; Lee, S.-H.; Endo, T. *Biomacromolecules* **2013**, *14* (7), 2420−2426.

(10) Bubner, P.; Plank, H.; Nidetzky, B. *Biotechnol. Bioeng.* **2013**, *110* (6), 1529−1549.

(11) Percival Zhang, Y.-H.; Himmel, M. E.; Mielenz, J. R. *Biotechnol. Adv.* **2006**, *24* (5), 452−481.

(12) Zhang, Z.; Donaldson, A. A.; Ma, X. *Biotechnol. Adv.* **2012**, *30* (4), 913−919.

(13) Martin-Sampedro, R.; Rahikainen, J. L.; Johansson, L. S.; Marjamaa, K.; Laine, J.; Kruus, K.; Rojas, O. J. *Biomacromolecules* **2013**, *14* (4), 1231−1239.

(14) Berlin, A.; Balakshin, M.; Gilkes, N.; Kadla, J.; Maximenko, V.; Kubo, S.; Saddler, J. *J. Biotechnol.* **2006**, *125* (2), 198−209.

(15) Sewalt, V. J. H.; Glasser, W. G.; Beauchemin, K. A. *J. Agric. Food Chem.* **1997**, *45* (5), 1823−1828.

(16) Nakagame, S.; Chandra, R. P.; Kadla, J. F.; Saddler, J. N. *Bioresour. Technol.* **2011**, *102* (6), 4507−4517.

(17) Rahikainen, J. L.; Martin-Sampedro, R.; Heikkinen, H.; Rovio, S.; Marjamaa, K.; Tamminen, T.; Rojas, O. J.; Kruus, K. *Bioresour. Technol.* **2013**, *133*, 270−278.

(18) Sammond, D. W.; Yarbrough, J. M.; Mansfield, E.; Bomble, Y. J.; Hobdey, S. E.; Decker, S. R.; Taylor, L. E.; Resch, M. G.; Bozell, J. J.; Himmel, M. E.; Vinzant, T. B.; et al. *J. Biol. Chem.* **2014**, *289* (30), 20960−20969.

(19) Fritz, C.; Ferrer, A.; Salas, C.; Jameel, H.; Rojas, O. J. *Biomacromolecules* **2015**, *16* (12), 3878−3888.

(20) Hoeger, I. C.; Filpponen, I.; Martin-Sampedro, R.; Johansson, L.-S.; Österberg, M.; Laine, J.; Kelley, S.; Rojas, O. J. *Biomacromolecules* **2012**, *13* (10), 3228−3240.

(21) Tammelin, T.; Johnsen, I. A.; Österberg, M.; Stenius, P.; Laine, J. *Nord. Pulp Pap. Res. J.* **2007**, *22* (1), 93−101.

(22) Constantino, C.; Dhanabalan, A.; Cotta, M.; Pereira-da-Silva, M.; Curvelo, A.; Oliveira, O. *Holzforschung* **2000**, *54* (1), 55−60.

(23) Constantino, C. J. L.; Juliani, L. P.; Botaro, V. R.; Balogh, D. T.; Pereira, M. R.; Ticianelli, E. A.; Curvelo, A. A. S.; Oliveira, O. N. *Thin Solid Films* **1996**, *284*−*285* (0), 191−194.

(24) Micic, M.; Benitez, I.; Ruano, M.; Mavers, M.; Jeremic, M.; Radotic, K.; Moy, V.; Leblanc, R. M. *Chem. Phys. Lett.* **2001**, *347* (1), 41−45.

(25) Notley, S. M.; Norgren, M. *Biomacromolecules* **2008**, *9* (7), 2081−2086.

(26) Tammelin, T.; Österberg, M.; Johansson, L. S.; Laine, J. *Nord. Pulp Pap. Res. J.* **2006**, *21* (4), 444−450.

(27) Morales, L. O.; Iakovlev, M.; Martin-Sampedro, R.; Rahikainen, J. L.; Laine, J.; van Heiningen, A.; Rojas, O. J. *Bioresour. Technol.* **2014**, *161*, 55−62.

(28) Salas, C.; Rojas, O. J.; Lucia, L. A.; Hubbe, M. A.; Genzer, J. *ACS Appl. Mater. Interfaces* **2013**, *5* (1), 199−206.

(29) Nakagame, S.; Chandra, R. P.; Saddler, J. N. *Biotechnol. Bioeng.* **2010**, *105* (5), 871−879.

(30) Björkman, A. *Sven. Papperstidn.* **1956**, *59* (13), 477−485.

(31) Capanema, E.; Balakshin, M.; Katahira, R.; Chang, H. M.; Jameel, H. *J. Wood Chem. Technol.* **2014**, *35* (1), 17−26.

(32) Crestini, C.; Melone, F.; Sette, M.; Saladino, R. *Biomacromolecules* **2011**, *12* (11), 3928−3935.

(33) Martin-Sampedro, R.; Capanema, E. A.; Hoeger, I.; Villar, J. C.; Rojas, O. J. *J. Agric. Food Chem.* **2011**, *59* (16), 8761−8769.

(34) Notley, S. M.; Norgren, M. *Langmuir* **2010**, *26* (8), 5484−5490.

(35) Rencoret, J.; Marques, G.; Gutierrez, A.; Nieto, L.; Jimenez-Barbero, J.; Martinez, A. T.; del Río, J. C. *Ind. Crops Prod.* **2009**, *30* (1), 137−143.

(36) Suurnäkki, A.; Tenkanen, M.; Siika-aho, M.; Niku-Paavola, M.-L.; Viikari, L.; Buchert, J. *Cellulose* **2000**, *7* (2), 189−209.

(37) Tappi Methods T222 om-88 (1988), "Acid-insoluble lignin in wood and pulp," and UM 250 (1985), "Acid-insoluble lignin in wood and pulp"; TAPPI PRESS, Atlanta, GA.
(38) del Rio, J. C.; Rencoret, J.; Prinsen, P.; Martinez, A. T.; Ralph, J.; Gutierrez, A. *J. Agric. Food Chem.* **2012**, *60* (23), 5922−5935.
(39) Ralph, S. A.; Ralph, J.; Landucci, L. L. *NMR Database of Lignin and Cell Wall Model Compounds*, 2009. www.glbrc.org/databases_and_software/nmrdatabase/ (accessed January 2017).
(40) Rencoret, J.; Gutiérrez, A.; Nieto, L.; Jiménez-Barbero, J.; Faulds, C. B.; Kim, H.; Ralph, J.; Martínez, Á.T.; del Rio, J. C. *Plant Physiol.* **2011**, *155*, 667.
(41) Lou, H.; Zhu, J. Y.; Lan, T. Q.; Lai, H.; Qiu, X. *ChemSusChem* **2013**, *6* (5), 919−927.
(42) Vörös, J. *Biophys. J.* **2004**, *87* (1), 553−561.
(43) Johannsmann, D.; Mathauer, K.; Wegner, G.; Knoll, W. *Phys. Rev. B: Condens. Matter Mater. Phys.* **1992**, *46* (12), 7808.
(44) Jung, L. S.; Campbell, C. T.; Chinowsky, T. M.; Mar, M. N.; Yee, S. S. *Langmuir* **1998**, *14* (19), 5636−5648.
(45) Shen, F. *Affinity Interaction between Hexamer Peptide Ligand HWRGWV and Immunoglobulin G Studied by Quartz Crystal Microbalance and Surface Plasmon Resonance*, Ph.D. Thesis, North Carolina State University, Raleigh, NC, USA, 2010.
(46) Hui, J. P.; Lanthier, P.; White, T. C.; McHugh, S. G.; Yaguchi, M.; Roy, R.; Thibault, P. *J. Chromatogr., Biomed. Appl.* **2001**, *752* (2), 349−368.
(47) Várnai, A.; Siika-aho, M.; Viikari, L. *Biotechnol. Biofuels* **2013**, *6* (1), 30.
(48) Pasquini, D.; Balogh, D.; Antunes, P.; Constantino, C.; Curvelo, A.; Aroca, R.; Oliveira, O. *Langmuir* **2002**, *18* (17), 6593−6596.
(49) Pu, Y.; Cao, S.; Ragauskas, A. J. *Energy Environ. Sci.* **2011**, *4* (9), 3154−3166.
(50) Johannsmann, D.; Mathauer, K.; Wegner, G.; Knoll, W. *Phys. Rev. B: Condens. Matter Mater. Phys.* **1992**, *46*, 7808−7815.
(51) Yu, Z.; Gwak, K. S.; Treasure, T.; Jameel, H.; Chang, H. M.; Park, S. *ChemSusChem* **2014**, *7* (7), 1942−1950.
(52) Suchy, M.; Linder, M. B.; Tammelin, T.; Campbell, J. M.; Vuorinen, T.; Kontturi, E. *Langmuir* **2011**, *27* (14), 8819−8828.
(53) Rahikainen, J. L.; Evans, J. D.; Mikander, S.; Kalliola, A.; Puranen, T.; Tamminen, T.; Marjamaa, K.; Kruus, K. *Enzyme Microb. Technol.* **2013**, *53* (5), 315−321.

DOI: 10.1021/acs.biomac.7b00071
Biomacromolecules 2017, 18, 1322−1332

VI. CONCLUSIONES

Paulownia (*Paulownia fortunei*)

En la presente Tesis se ha evaluado el uso del sistema lacasa-mediador como pretratamiento para eliminar/modificar la lignina presente en residuos agrícolas (paja de trigo, el bagazo y la paja de la caña de azúcar) y cultivos de crecimiento rápido (paulownia), con el objetivo de conseguir un aprovechamiento más eficaz y racional de dichos materiales, incluyendo la producción de bioetanol de segunda generación. Además se han estudiado las interacciones entre la lignina y las celulasas usadas en la sacarificación con el fin de demostrar el efecto que tiene la presencia de la misma en la hidrólisis del material lignocelulósico. Las principales conclusiones obtenidas de los estudios llevados a cabo se citan a continuación:

1. El pretratamiento enzimático de la paja de trigo con la lacasa de *Pycnoporus cinnabarinus* y HBT como mediador redox, seguido de una extracción alcalina con peróxido de hidrógeno, demostró una gran eficacia en cuanto a la deslignificación de esta materia prima, obteniéndose un descenso de lignina del 45% y una mejora en la obtención de glucosa tras sacarificación enzimática del 60%.

2. El pretratamiento con la lacasa de *P. cinnabarinus* y HBT (consistente en 4 ciclos de tratamiento enzimático seguidos de extracción alcalina) mostró una gran eficacia para la deslignificación de los residuos de caña de azúcar, con un descenso del 27% en el caso del bagazo y de un descenso del 31% en el caso de la paja, consiguiendo a su vez una mejora del 39% y 46% en el rendimiento de glucosa, respectivamente, tras sacarificación enzimática.

3. El pretratamiento de la madera de paulownia con la lacasa comercial de *M. thermophila* y siringato de metilo como mediador redox (en 4 ciclos, seguido de la correspondiente extracción alcalina) consiguió reducir el contenido en lignina en un 25%, y produjo una mejora en la sacarificación de un 40%. El análisis de los filtrados obtenidos en los pretratamientos reveló la presencia de compuestos oxidados de bajo peso molecular derivados de la lignina (aldehídos como vainillina y siringaldehído, y ácidos como ácidos vanílico y siríngico), especialmente en los filtrados obtenidos

después del pretratamiento enzimático, confirmando por tanto la despolimerización de la lignina.

4. Los análisis 2D-NMR de la paja de trigo, el bagazo y la paja de la caña de azúcar y de paulownia, tras los diversos pretratamientos enzimáticos, revelaron en todos los casos una eliminación significativa de las diferentes unidades de lignina, tanto las de tipo siringilo como las de tipo guayacilo y *p*-cumarilo, así como un aumento de las unidades oxidadas de siringilo y guayacilo, sumado a una ruptura de los enlaces éter β-*O*-4. Estos resultados demostraron que el pretratamiento enzimático consiste en una despolimerización oxidativa de la lignina.

5. El estudio de las interacciones lignina-celulasas, tanto en mezclas comerciales como purificadas (exoglucanasa de *Trichoderma reesei*), demostró que la enzima purificada presentaba una menor afinidad por la lignina y una mayor reversibilidad a dicha unión. Los estudios mediante QCM y SPR demostraron la importancia de las interacciones electrostáticas en esta adsorción. A su vez se observó que el nivel de adsorción estaba relacionado con la relación entre unidades de lignina de tipo siringilo y de tipo guaiacilo (relación S/G), así, el mayor valor de adsorción correspondía a la lignina de pícea, con un 99% de unidades de tpo G, mientras que la adsorción más baja se produjo con la lignina de eucalipto, con un 70% de unidades de tipo S.

En conclusión, los pretratamientos enzimáticos basados en el sistema lacasa-mediador presentan un gran efectividad para la eliminación/modificación del polímero de lignina presente en los materiales lignocelulósicos, mejorando el rendimiento de la sacarificación y consecuentemente la producción de bioetanol de segunda generación. Por otro lado, se ha demostrado que la presencia del polímero de lignina tiene un efecto negativo en la hidrólisis de estos materiales, debido a la inhibición/adsorción de las celulasas, y que el alcance de esta depende en gran parte de la composición de la lignina del material lignocelulósico.


IRNAS
CSIC
UNIVERSIDAD DE SEVILLA